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**The effects of fatty acids and antioxidants on U937  
monocytes used as a model for the lipid disturbance  
in schizophrenia.**

**OLUWAKEMI OBAJIMI**

A thesis submitted in partial fulfillment of the requirements of the Open University for the  
degree of Doctor of Philosophy.

Sponsoring establishment, UHI Millennium Institute.

Academic partners, Scottish Association for Marine Science (SAMS) and

Highland Psychiatric Research Foundation (HPRF).

Biotechnology

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## **DEDICATION**

**To my parents**

## ABSTRACT

Schizophrenia is a devastating mental disease affecting approximately 1% of all populations. Recent studies have suggested that deficient uptake of free fatty acids or excessive breakdown of cell-membrane phospholipids, directly or indirectly triggered by lipid peroxidative damage, may be associated with schizophrenia and possibly other neurodegenerative disorders.

This study provides more information on the influence of the presence of different fatty acids (FAs): stearic acid (SA), oleic acid (OA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) and dietary antioxidants (ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin) as components of the culture-medium on the human monocytic cell line (U937) by the measurements of cell-viability, productions of by-products of lipid peroxidation (lipid hydroperoxides and volatile hydrocarbons), AA-release/uptake and cPLA<sub>2</sub> expression upon induced-oxidative stress with the radical-mediating system of t-butyl hydroperoxide (t-BHP)/Fe<sup>2+</sup>.

Of all FAs used, the PUFAs (AA, EPA and DHA) at 40  $\mu$ M concentrations reduced cell-membrane integrity significantly whereas SA and OA had no effect after 72 h of incubation, suggesting the cytotoxicity of the PUFAs above certain concentrations.

This study shows that U937 cells accumulate lipid hydroperoxides, generate ethane, butane and pentane, release and incorporate AA at a greater rate in response to induced-oxidative stress by a mechanism probably involving cPLA<sub>2</sub>. In addition, AA is incorporated into cellular phospholipids at a greater rate post-oxidation. With oxidative-stress, the presence

of EPA but not DHA in cellular phospholipids significantly increases the accumulation of AA in the extracellular fluid.

Studies revealed that under oxidative-stress  $\alpha$ -tocopherol is a potent antioxidant markedly inhibiting AA-release but not its uptake. Whilst ascorbic acid appeared to promote AA-release,  $\beta$ -carotene and astaxanthin conferred no protective effect. Collectively, this study demonstrates the potency of  $\alpha$ -tocopherol, ascorbic acid,  $\beta$ -carotene and astaxanthin against oxidant-induced peroxidative damage, AA release/uptake and ultimately total loss of cell-viability. In EPA-pretreated cells, AA-release is enhanced with/without oxidant-activation suggesting its action by stimulating the availability of AA possibly for prostaglandin production.

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## LIST OF ABBREVIATIONS USED

AA	-	Arachidonic acid
ANOVA	-	Analysis of variance
ATD	-	Automatic thermal desorption
ATP	-	Adenosine Triphosphate
Ax	-	Astaxanthin
BHT	-	Butylated hydroxy -toluene
BSA	-	Bovine serum albumin
CAT	-	Catalase
CI(s)	-	Confidence interval(s)
C: M	-	Chloroform: methanol
Ca <sup>2+</sup>	-	Calcium ion
cAMP	-	Cyclic adenosine monophosphate
CM	-	Culture medium
CO <sub>2</sub>	-	Carbondioxide
cPLA <sub>2</sub>	-	Cytosolic phospholipase A <sub>2</sub>
DAG	-	Diacylglycerol
DHA	-	Docosaheptaenoic acid
ELISA	-	Enzyme-linked immunosorbent assay
EPA	-	Eicosapentaenoic acid
EtOH	-	Ethanol
FA(s)	-	Fatty acid(s)
FAME	-	Fatty acid methyl ester
FBS	-	Foetal bovine serum
FFA(s)	-	Free fatty acid(s)

FOX	-	Ferrous oxidation/Xylenol orange
GSHPx	-	Glutathione peroxidase
HBSS	-	Hanks balanced salt solution
iPLA <sub>2</sub>	-	Intracellular phospholipase A <sub>2</sub>
LDH	-	Lactate dehydrogenase
LPC	-	Lysophosphatidyl choline
LPL(s)	-	Lysophospholipid(s)
NAD <sup>+</sup>	-	Nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dinucleotide (reduced form)
O <sub>2</sub>	-	Oxygen
OA	-	Oleic acid
PAF(s)	-	Platelet activating factor
PC	-	Phosphatidylcholine
PE	-	Phosphatidylethanolamine
PI	-	Phosphatidylinositol
PLA <sub>2</sub> (s)	-	Phospholipase(s)
PS	-	Phosphatidylserine
PUFA(s)	-	Polyunsaturated fatty acids
SA	-	Stearic acid
SOD	-	Superoxide dismutase
sPLA <sub>2</sub>	-	Secretory phospholipase A <sub>2</sub>
t-BHP/Fe <sup>2+</sup>	-	Tert-butyl hydroperoxide/iron sulphate
TLC	-	Thin layer chromatography

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## CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

### 1.1 GENERAL INTRODUCTION

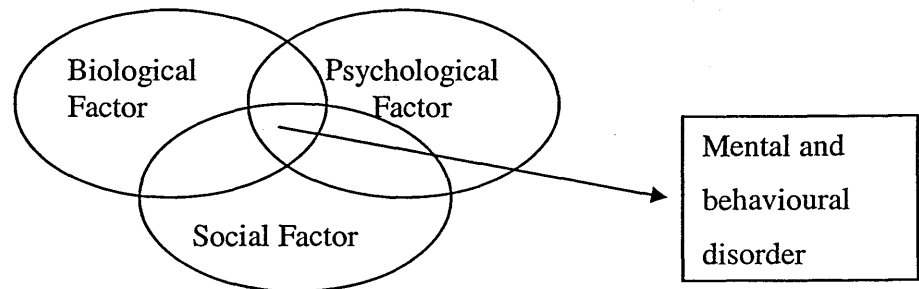
Schizophrenia is a psychiatric disease found to be common in all known populations with a lifetime prevalence of somewhere between 0.5% and 1.5% (WHO, 1979). The disorder normally strikes in young adulthood with a combination of hallucinations, delusions and social withdrawal symptoms (Strauss, 1994), frequently resulting in the unemployment of the individual thus costing the society a productive citizen. Studies estimate the overall UK cost burden of schizophrenia, including both direct and indirect costs, at £2.6 billion pounds sterling per annum in 1997 (Knapp, 1997). For the past 40 years, the dominant theme governing research into schizophrenia, the affective disorders such as tardive dyskinesia, and other central nervous system diseases, has related these conditions to neurotransmitter system abnormalities especially those affecting dopamine D2 receptors. As a result, therapeutics, largely developed in the 1950s, have been directed towards finding agents that will modulate the neurotransmitter system in some way (Horrobin 1999a). However, recent studies using receptor labels *in vivo*, have failed to show any consistent abnormality of dopamine D2 receptors in drug naive schizophrenic patients (Farde 1997). Apart from that, the current antipsychotic drugs have only limited efficacy in many patients and possess numerous side effects such as tardive dyskinesia and obesity, which limit patient compliance (Kane 1994). There is, therefore, the need for improvements in the treatment of schizophrenia, which will have significant payoffs in both the human and financial costs of the disorder. Over the past two decades a large number of studies have examined a hypothesis with direct implications for the treatment of schizophrenia: "the membrane phospholipid hypothesis" (Horrobin et al., 1994). The hypothesis states that a systemic abnormality in one or more aspects of membrane phospholipid metabolism is an etiological factor in the development and/or symptoms of schizophrenia. Interest in this area of research has steadily increased characterized by the

recent review by Fenton and colleagues (Fenton et al., 2000) who state that “Studies of fatty acid metabolism have proved fruitful for generating and testing novel etiologic hypotheses and new therapeutic agents for schizophrenia”. Furthermore, treatment of schizophrenia with lipids and their derivatives, in particular the fatty acid eicosapentaenoic acid (EPA) but not docosahexaenoic acid (DHA), has produced promising preliminary results and is the subject of several ongoing clinical trials in the USA and Europe (Richardson et al., 1999; 2000; Joy et al., 2000; Puri et al., 2000). A key piece of evidence in support of a membrane abnormality in schizophrenia comes from experiments utilizing the B-vitamin niacin (nicotinic acid). That study demonstrated that vasodilatory response to niacin is deficient in schizophrenia (Ward et al., 1998). The vasodilatory response is dependent upon the mobilization of arachidonic acid in skin macrophages and its conversion to prostaglandins. It is also a marker of the availability of arachidonic acid for cell signaling. The phenomenon indicates that the availability of arachidonic acid is reduced in schizophrenia (Olney and Farber, 1995).

## **1.2 NEURODEGENERATIVE DISEASES**

The world now suffers from an increasing burden of mental illnesses with a rising need for improvements in treatments. At present, about 450 million people suffer from mental or behavioural disorders, with only a small proportion receiving even the most basic treatment (WHO 2001). In most developing countries, many individuals with severe neuronal disorders are left to cope as best as they can with their personal burdens. In all populations, many are victimized for their illness becoming targets of stigma and discrimination. Globally, neuro-psychiatric illnesses represent four of the ten leading causes of disability (WHO, 2001). This increasing burden results in high costs in terms of human and

economic loss. It is now known that most illnesses, both mental and physical, are influenced by a combination of biological, psychological and social factors.



Mental and behavioural disorders affect people of all ages, races, sexes, in all countries and they cause sufferings to families, communities and the individuals themselves.

- Biological factors include age and sex. These are associated with mental and behavioural disorders. For example, depression has been shown to be sex-related probably due to hormonal changes, domestic and sexual violence more common to women (WHO, 2001).
- Psychological factors: One of the main findings throughout the 20<sup>th</sup> century that has shaped current understanding is the importance of relationships with parents or guardians during childhood. Affectionate, attentive and stable caring allows infants and young children to normally develop such functions as language, intellect and emotional regulation. Children deprived of nurture from parents or guardians are more likely to develop mental and behavioural disorders, either during childhood or later in life irrespective of adequate nutrition and bodily care. Secondly, human behaviour is partly shaped through interactions with the natural or social environment, which can result in either desirable or undesirable consequences. Most individuals engage in rewarding activities while others engage in ones inviting punishment. Mental and behavioural disorders can be seen as maladaptive

behaviour that has been learned either directly or through observing others over time (WHO, 2001). Thirdly, mental and behavioural disorders such as anxiety and depression can occur from the failure of an individual to cope adaptively to stressful events of life. Generally, people who try to avoid thinking about or dealing with stressors are more susceptible to anxiety and depression. On the other hand, those who share their problems attempting to find ways of managing stressors, function better over time (WHO, 2001).

- Social factors: For example, urbanization, racism, poverty and technological change have been associated with the development of mental and behavioural disorders. Urbanization may contribute to this disorder through the influence of increased stressors and adverse life events such as overcrowding, polluted environments, poverty or dependence on a cash economy, high violence levels and reduced social support. Rural life also imposes problems on people. For example, isolation, lack of transport and communication, limited education and economic opportunities. Poverty is linked with economic deprivation, low education and unemployment.

### **1.3 SCHIZOPHRENIA**

Schizophrenia is a severe mental disorder that typically begins in late adolescence or early adulthood. From the clinical perspective, signs and symptoms are many, varied and are often classified as “positive” and “negative” symptoms. The positive symptoms refer to the hallucinations, delusions (strong belief in ideas that are false and without basis in reality), thought alienation and disorder which typically occur early on in the course of the illness and which respond well to treatment. Negative Symptoms include emotional blunting, social withdrawal, cognitive deficits and poverty of speech and activity (Kuperberg and Heckers, 2000). Schizophrenia is found to exist approximately equally between males and

females though the onset tends to be later in females. The economic cost of schizophrenia to society is very high. In 1991 an estimate of the cost burden of schizophrenia in the United States was US\$ 19 billion in direct expenditure and US\$ 46 billion in lost productivity (WHO, 2001). It is the third most costly illness to the Canadian economy, estimated to be \$2.35 billion dollars in 1996 (Goeree et al., 1999).

### **1.3.1 Treatment of schizophrenia**

The discovery and improvement of useful therapeutics for the management of schizophrenia occurred largely in the second half of the 20<sup>th</sup> century. The first being chlorpromazine, was developed from compounds with anti-histaminic and sedative properties. It was originally used as part of a pre-anesthetic cocktail, which was found to induce a state of mind similar to that observed in schizophrenic patients after treatment by frontal lobotomy, a popular treatment for schizophrenia at that time (Horrobin, 1999a). Chlorpromazine (an antipsychotic drug) was thought to act by blocking dopamine D2 receptors (Zimmer et al, 1998) therefore, the development of drugs in the 1950s was directed at finding agents that will modulate these receptors in some way. However recent studies have failed to show any consistent dysfunction in dopamine D2 receptors (Farde, 1997). In addition, after the disappearance of the obvious symptoms of schizophrenia, some residual symptoms may remain. These include lack of interest and initiative in daily activities and work, social incompetence and inability to take interest in pleasurable activities possibly causing continued disability and poor life quality to the individual and placing considerable burden on families (Pai and Kapur, 1982).

However, it has been repeatedly demonstrated that schizophrenia follows a less severe course in developing countries (Kulhara and Wig 1978; Thara and Eaton 1996). For

instance, the proportion of patients showing full remission at 2 years was 63% in developing countries compared to 37% in developed countries (Jablensky et al., 1992). Possible explanations for this better outcome are based on stronger family support and fewer demands on the patients, although the exact reasons for these differences are unclear. Schizophrenia reduces an affected individual's life span by an average of 10 years worldwide.

Currently, the primary prevention of schizophrenia is impossible. Treatment of schizophrenia has 3 main components: medications to relieve symptoms and prevent relapse; education and psychosocial interventions to help patients and their families to cope with the illness and its complications and help prevent relapse; rehabilitation to help patients back into the community and regain educational and occupational functioning. The ultimate goals of treatment are to identify the illness as soon as possible, treat symptoms, provide skills to patients and their families, maintain improvement over time, prevent relapse, re-integrate patients into the community, and to regain normal life. Presently, two groups of drugs are used in the treatment of schizophrenia.

Standard antipsychotics (also referred to as neuroleptics): This group of drugs was introduced 50 years ago and have proved useful in decreasing, and sometimes eliminating, certain symptoms of schizophrenia such as hallucinations and delusions. These drugs function by blocking dopamine D2 receptors in the brain thus reducing the overactivity of dopamine. Dopamine is the chemical messenger in the brain mainly involved with thinking, emotions, behaviour and perception. However, recent studies using receptor labels in vivo showed no consistent abnormalities in dopamine D2 receptors in drug-naive schizophrenics (Farde, 1997). They are also known to reduce associated symptoms such as



agitation, impulsiveness and aggressiveness. This can be achieved in days or weeks in about 70% of patients. If these drugs are consistently used, they can also reduce the risk of relapse by up to 50% (WHO, 2001). Antipsychotic drugs address the symptoms of the disease and not the disease itself nor its cause. They are therefore not curative but rather drugs to reduce or control symptoms or to prevent relapse of the disease. Besides, these drugs have been shown to lose their potency in a significant number of patients over a period of time. Furthermore, after chronic treatments with these drugs, some patients develop side effects such as movement disorders, including drug-induced Parkinsonism, tardive dyskinesia (TD), akathisia (a subjective sense of restlessness) and cardiovascular and cognitive effects (American College of Neuropsychopharmacology, 1973; Kane and Smith, 1982; Yassa et al., 1997) causing extreme discomfort to patients resulting in medication non-compliance and relapse of the disease (Kane, 1994).

Novel antipsychotics (also known as “atypical” antipsychotics): These newer drugs have less side effects but there is no clear evidence that they differ significantly from the older drugs in their effectiveness. Most recent economic studies have focused on the cost-effectiveness of active pharmacotherapies indicating that the newer drugs are not significantly more efficacious than the older ones though they possess fewer side-effects.

There therefore, seems to be a knowledge gap in the cause of the disease and a loophole in the efficacy and effectiveness of the available pharmacotherapies, which is thought to be explained by the membrane phospholipid hypothesis of schizophrenia (Horrobin, 1999a) as will be discussed in this thesis.

#### **1.4 NEURODEVELOPMENTAL APPROACH TO SCHIZOPHRENIA**

A neurodevelopmental approach to schizophrenia proposes that interaction between genetic and early environmental factors affects both the formation process and functions of nerve cells (Nasrallah, 1993; Falkai and Bogerts, 1995; Weinberger 1995). These factors start operating during the neurodevelopmental stages of the foetus, influenced by perinatal events. The factorial effects become fully expressed in early adulthood when the brain finally matures with completed myelination.

Some key pieces of evidence for a neurodevelopmental approach are:

- Abnormalities in brain morphology and an increased ventricular size in schizophrenic patients (Nasrallah, 1993; Falkai and Bogerts, 1995; Weinberger, 1995). Some of these deviations from normal occur well before the development of overt schizophrenic symptoms (Horrobin, 1999b).
- Behavioural and neuromotor abnormalities occur in childhood, which may be predictive to some degree of later schizophrenia. These abnormalities include low IQ (David et al., 1997); poor motor skills (Marcus, 1974; Jones, 1994); poor language and word skill development (Crow 1995; 1996); lack of concentration (Rieder and Nichols 1979) and poor social development (Fish 1987). In many people who later develop schizophrenia, certain behavioural patterns are detectable in childhood and adolescence (Horrobin, 1999b).
- During pregnancy and the perinatal period, events such as obstetric complications, low birth weight and perinatal hypoxia (McNeil and Kaij, 1978; O'Callaghan et al., 1992; McGrath and Murray, 1995); starvation during pregnancy (Susser and Lin, 1992); low head circumference in otherwise normal individuals (McNeil et al., 1993); viral infections especially in the second trimester (O'Callaghan et al., 1991;

Sham et al., 1992), have been associated with an increased risk of later schizophrenia. On the other hand, prolonged breast-feeding appears to be protective against the later development of schizophrenia (McCreadie, 1997; Peet, 1997).

- There are sex differences at the onset and clinical course of the disease, with females usually affected later, and less severely than males but showing a second, late post-menopausal, peak in incidence (Iacano, 1992; Murray et al., 1992).
- In both hemispheres, the risk of schizophrenia appears to be higher in children born during winter months than those at other times (Dalen, 1975; Yakley and Murray, 1995).

These findings suggest that there may be a genetic and environmental basis for schizophrenia. However, none of these neurodevelopmental influences seems to individually account for more than a small percentage of the total number of schizophrenic patients in the community at large. There is still a fundamental lack of biochemical explanation for the expression of the genetic and neurodevelopmental influences in schizophrenia. Hypofunction of glutamate receptors has been put forward as a biochemical substrate for developmental problems, but this does not fully explain all the known neurodevelopmental effects (Olney and Farber, 1995). The observations consistent with the neurodevelopmental hypothesis are thought to occur by a disturbed phospholipid metabolism (Horrobin et al., 1994; Mahadik and Evans, 1997; Horrobin 1998), which can be explained by the “membrane phospholipid hypothesis”.

## **1.5 MEMBRANE PHOSPHOLIPID HYPOTHESIS OF SCHIZOPHRENIA**

Over the past two decades a large number of studies have examined a hypothesis with direct implications for the treatment of schizophrenia, “the membrane phospholipid

hypothesis” (Horrobin et al., 1994). This hypothesis states that “a systemic abnormality in one or more aspects of membrane phospholipid metabolism is an etiological factor in the development and/or symptoms of schizophrenia”. The phospholipid hypothesis proposes that “in schizophrenia, there are at least two primary faulty genetic abnormalities and that the effect of these may be attenuated or exacerbated by a third gene. The two primary abnormalities are an increased rate of removal of polyunsaturated fatty acids (PUFAs), from membrane phospholipids, coupled with a reduced rate of incorporation of these same fatty acids into membrane phospholipids” (Horrobin, 1998). Each of these abnormalities may arise from changes in the activity or regulation of one or more of a group of phospholipase enzymes. Horrobin et al. (1994) suggested that schizophrenia is a disorder with abnormal phospholipid structure and metabolism, not only in neuronal cells but also in other tissues. Several studies on schizophrenic patients have reported various findings supporting the membrane phospholipid hypothesis:

- Reports have shown depleted levels of several polyunsaturated fatty acids, especially arachidonic acid (AA) in phospholipids from red blood cell membranes of schizophrenic patients (Ramchand, 1996).
- Treatment of schizophrenia with essential fatty acids and their derivatives, in particular the polyunsaturated fatty acid EPA, has yielded positive preliminary results and is the subject of ongoing clinical trials in Europe and North America (Richardson et al., 1999; Fenton, 2000; Joy et al., 2000; Richardson et al, 2000; Puri et al., 2000).
- Schizophrenics’ show reduced flushing after oral or topical administration of the B-vitamin, niacin (Ramchand et al., 1996; Ward et al., 1998), suggesting that schizophrenics have reduced availability of AA as flushing after niacin

administration occurs upon the enhancement of the production of prostaglandins from AA. This response is a marker of AA available for cell signaling.

- Glutamate receptor hypofunction proposed by Olney and Farber (1995), as a biochemical substrate for developmental problems can even be explained by the membrane phospholipid hypothesis, since normal functioning of glutamate receptors require normal AA levels in membrane phospholipids (Horrobin, 1999b).
- There is considerable evidence that schizophrenic cells show increased levels of lipid peroxidation. Studies have demonstrated increased hydrocarbon levels in expired air from schizophrenics (Hinsberger et al., 1995), and increased thiobarbituric acid reactive substances (TBARS) in drug naive patients during their first psychotic episodes (Ramchand et al., 1996). Hydrocarbons and TBARS are biomarkers of lipid peroxidation.
- Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) has been reported to be increased in platelets from schizophrenic patients (Ramchand et al., 1996). Also, <sup>31</sup>P magnetic resonance imaging has shown evidence of an increase in phospholipid breakdown in schizophrenia (Yao et al., 2002), which might be as the result of an over-active PLA<sub>2</sub> (Glen et al., 1994; Hinsberger et al., 1995).
- Previous studies of antioxidant enzyme activity levels in schizophrenic patients have generated controversial results. Some studies have reported a significant increase in superoxide dismutase (SOD) activity levels (Abdalla et al., 1986; Reddy et al., 1991; Lohr, 1991; Kuloglu et al., 2002) and high GSHPx levels (Kuloglu et al., 2002). Others have shown reduced GSHPx levels (Abdalla et al., 1986; Stoklasova et al., 1986) and low catalase (CAT) activity in schizophrenic patients compared to controls (Reddy et al., 1991). However

many of these reports suggest a decrease in cellular protection against oxidative damage to membrane phospholipids.

- Clinical trials for three months with PUFA supplementation in schizophrenic patients showed significant improvements in schizophrenic symptoms with EPA treatments in comparison to patients treated with DHA-supplements (Peet et al., 2001).

## **1.6 BIOLOGICAL CELL MEMBRANES**

Biological membranes are organized sheet-like assemblies consisting mainly of lipids and proteins. These sheet-like structures are non-covalent assemblies, thermodynamically stable and metabolically active. Membrane functions are indispensable for life. Plasma membranes give cells their integrity by separating them from their environment. Cell membranes are highly selective permeability barriers rather than impervious walls since they contain specific molecular pumps and gates, used for the transport of essential molecules and ions. Eukaryotic cells also contain internal membranes forming the boundaries of organelles such as the mitochondria, chloroplast, lysosomes, peroxisomes etc.

Membranes control the flow of information between cells and their environment. They contain specific receptors for external stimuli. For example, the movement of bacteria towards food, the response of target cells to hormones such as adrenalin, and light perception are processes in which the primary event is the detection of a signal by a specific membrane receptor. In turn, some membranes generate signals, which can be chemical or electrical. Thus, cell membranes play a critical and central role in biological communication both hormonal- and neuro-transmission.

Far from being just a bag of soluble components, the cell is viewed as a highly organized entity with many functional compartments each limited by one or more membranes. Membranes are highly viscous yet plastic structures surrounding all living cells. Plasma membranes form closed compartments around cellular protoplasm separating one cell from another, permitting cellular individuality. The plasma membrane has selective differences in composition between the internal and external cellular environments. The selective permeability is provided by pumps and channels for ions and substrates by specific receptor signals.

### **1.6.1 The importance of biological membranes**

Alterations of membrane structures can affect water balance, ion influx and outflux and therefore, all cellular processes. Specific deficiencies or alterations of certain membrane components lead to a variety of diseases. For example, lack of an iodide transporter, causes congenital goitre. The maintenance of a normal environment around and in a cell is a fundamental requirement. Water makes up about 56% of the lean body mass of the human body and is distributed in two large compartments namely the intracellular and extracellular fluids. The intracellular fluid (ICF) compartment constitutes two-thirds of total water and provides the environment for the cell to make, store and utilize energy, repair itself, replicate and perform special functions. On the other hand, the extracellular fluid (ECF) contains a third of the total body water and is distributed between the plasma and interstitial compartments. The ECF is a delivery system. It provides to the cell nutrients such as glucose, fatty acids, amino acids, vitamins, various ions, trace minerals and a variety of respiratory molecules (hormones) that coordinate the functions of widely separated cells. The ECF removes carbon-dioxide waste products, toxic and detoxified materials from the immediate cellular environment. Common features of biological membranes are:

- Membranes are sheet-like structures with a thickness between 60 and 100 Å.
- They consist mainly of proteins and lipids. In most biological membranes, the weight ratio of protein to lipids ranges from 1:4 to 4:1. They also contain carbohydrates linked to lipids and proteins.
- Membrane lipids are relatively small molecules possessing both a hydrophilic and hydrophobic moiety. These lipids spontaneously form closed bimolecular sheets in aqueous media (Fig. 1.1). The lipid bi-layers form barriers to the flow of polar molecules.
- Membrane proteins are specific in nature. They mediate distinctive functions of membranes. They function as pumps, gates or channels, receptors, energy transducers and enzymes. They are embedded in lipid bi-layers, which create suitable environments for their action.
- Cell membranes are non-covalent assemblies i.e. the constituent lipid and protein molecules are held together by many non-covalent interactions, which are cooperative.
- Membranes are asymmetric i.e. the two faces of a membrane are different.
- Membranes are fluid structures. Lipid molecules diffuse rapidly in the plane of the membrane, as do proteins, unless they are anchored by specific interactions but they do not rotate across the membrane. Membranes can be regarded as two-dimensional solutions of oriented proteins and lipids.



**Table 1.1**      The protein : lipid ratio of some purified membranes

<u>Percentage by Weight</u>			
<u>Membrane</u>	<u>Protein</u>	<u>Lipid</u>	<u>Carbohydrate</u>
Myelin	18	79	3
Plasma Membrane:			
Human RBC	49	43	8
Mouse Liver	44	52	4
Amoeba	54	42	4
Mitochondrial Liver	76	24	0
Membrane			

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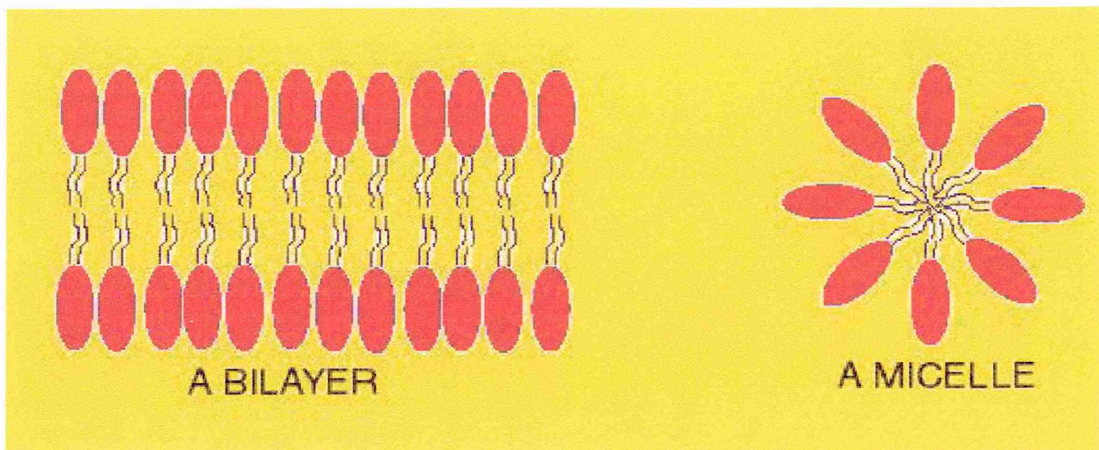
## 1.7      MEMBRANE LIPIDS

Membrane lipids are sheet like structures, only a few molecules thick, that form closed boundaries between compartments of different composition. Lipids are water in-soluble bio-molecules that are highly soluble in organic solvents such as chloroform. They have a wide variety of biological functions including serving as fuel molecules, highly concentrated energy stores, signal molecules and components of the membranes. There are different classes of lipids but all derive their distinctive properties from the hydrocarbon nature of a major portion of their structure. Lipids serve as protective coatings on the surface of many organisms and also play significant roles in cell recognition, species specificity and tissue immunity.

Lipids have fundamental structural and functional roles in cell membranes generally and particularly in the central nervous system. They form the matrix within which membrane

proteins and carbohydrates interact. Membrane proteins include receptors, ion channels and second messenger binding proteins. Second messengers are intracellular mediators formed by the binding of an effector to a receptor in cell membranes. Membranes differ in their protein content depending on their functions. For example, myelin, a membrane serving as an insulator around certain nerve fibers, is low in protein (18%) with lipids being the major molecular species. A dysfunction in membrane lipids may therefore result in defective membrane protein activities, cellular ion transport and may even result in cell death. So far, lipids have been largely ignored and the neurochemical basis for most psychiatric disorders concentrated on, with particular emphasis primarily on neurotransmitters and their receptor systems (Peet, et al., 1999). However, more recently, there has been increasing recognition that neurotransmitters and the receptor mechanisms are substantially influenced by the lipid environment without which there will be profound disorders in membrane activities (Witt and Nielson, 1994).

Membrane lipids constitute about 50% of the mass of most animal cell plasma membranes. All are amphipathic molecules containing both hydrophilic and hydrophobic surfaces. They can take the shape of a bilayer (bimolecular sheets with the hydrophobic tails sandwiched between the hydrophilic head groups) or a micelle (a spherical shape with tails pointing inward) (Fig. 1.1). Membrane lipids have many functions including the control of cell permeability and fluidity.



**Fig. 1.1** Phospholipid bilayer

Unlike plasma membranes of non-neuronal cells, neuronal plasma membranes, contain as much as 69.5% of the total cell lipids, constituting over 50% of the dry weight in human brain, are enriched in respect of four major phospholipid classes: phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine (Suzuki et al., 1981). As much as 20% of the brain by weight consists of PUFAs mostly AA and DHA (Horrobin, 1996) which are highly susceptible to oxidative damage.

## **1.8 MEMBRANE LIPID CLASSES**

There are three major kinds of membrane lipids, namely: phospholipids, glycolipids and cholesterol. Phospholipids are the major class of membrane lipids. They are abundant in all biological membranes.

**Table 1.2**      Lipids in animal cells

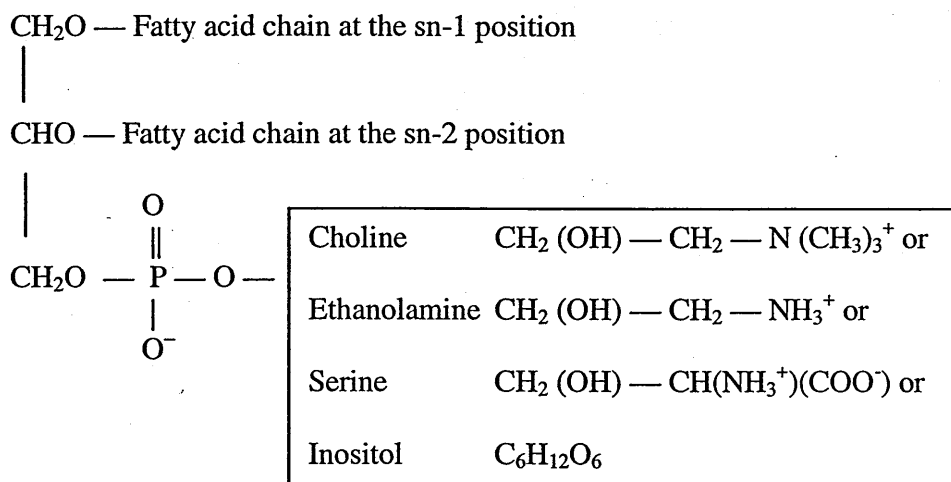
Lipid Class	Hydrophilic Unit	Hydrophobic Unit
Phospholipids:		
Phosphoglycerides	Phosphorylated alcohol	Fatty Acid chains
Sphingomyelin	Phosphoryl choline	Fatty acid chain and hydrocarbon chain of sphingosine
Glycolipid	One or more sugar Residues.	Fatty acid chain and hydrocarbon chain of sphingosine.
Cholesterol	OH group at C-3	Entire molecule excluding the hydroxyl group at C-3.

### **1.8.1 Phospholipids**

The two major phospholipid groups are phosphoglycerides (also known as glycerophospholipids) and sphingolipids composed of sphingomyelins.

#### **1.8.1.1 Phosphoglycerides**

They are the more common group of phospholipids. They are made up of a glycerol backbone attached via an ester linkage to two fatty acid chains and a phosphorylated alcohol (Fig. 1.2).



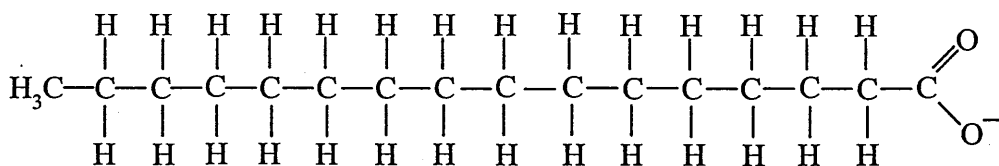
**Fig. 1.2** Phospholipid structure.

In glycerophospholipids, the hydrophilic unit is also known as the polar head group represented by a circle whereas, the hydrophobic unit containing the hydrocarbon tails are depicted by straight or wavy line (Fig. 1.1). In an aqueous medium, the polar head groups have the affinity for water whilst the hydrocarbon tails avoid water. These preferences are accommodated by the formation of a micelle, a globular structure in which polar head groups are on the surface in contact with the aqueous environment with the hydrocarbon tails sequestered inside (Fig. 1.1).

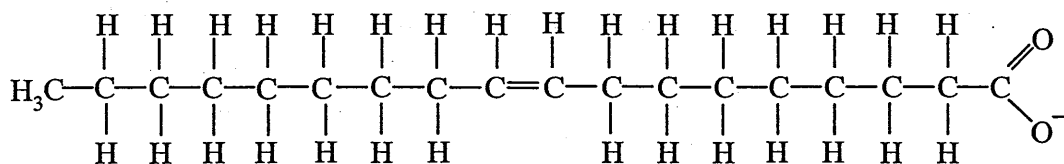
Another arrangement fulfilling both the hydrophilic and hydrophobic preferences of membrane lipids is a bimolecular sheet also known as the lipid bi-layer (Fig. 1.1). The favoured arrangement of phospholipids in an aqueous environment is the bi-layer form rather than a micelle as their two fatty acyl chains are too bulky to fit into the interior of a micelle. In contrast, salts of fatty acids containing only one fatty acyl chain readily form micelles. The formation of the bi-layer rather than the micelles by phospholipids is of critical importance in biological membranes. Whilst a micelle is a limited structure, usually

less than 200 Å in diameter, the lipid bi-layer can have macroscopic dimensions (millimeter,  $10^7$  Å). Phospholipids and glycolipids are important membrane constituents because they readily form extensive bimolecular sheets. In addition, these sheets serve as permeability barriers and yet are fluid in nature.

The formation of a lipid bi-layer is a self-assembly process, inherent in the structure of the lipid molecules in their amphipathic character. It is a rapid and spontaneous process in aqueous media driven predominantly by hydrophobic interactions. Water molecules are released from the hydrophobic tails of membrane lipids as these tails become sequestered in the non-polar interior of the bi-molecular sheet. van der Waals forces of attraction between the hydrophobic tails favour close packing. There are also electrostatic and hydrogen-bonding attractions between the polar head groups and water molecules. The lipid bi-layers are stabilized by a full array of forces that mediate molecular interactions in biological systems. In eukaryotic cells, the fatty acid constituents are usually even numbered carbon molecules, mostly containing 12 to 24 carbons. They are unbranched in animal cells and can be saturated or unsaturated. The double bond configuration in unsaturated fatty acids is almost always cis. The length and the degree of unsaturation in fatty acid chains in membrane lipids have profound effects on membrane fluidity.



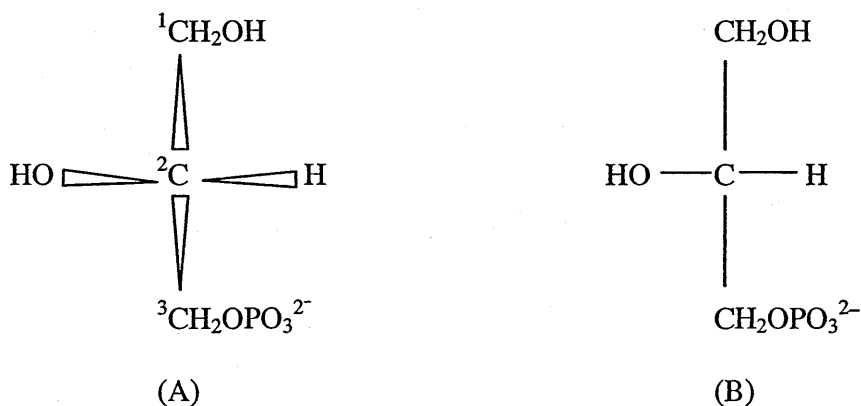
Palmitate (ionized form of palmitic acid) ( $\text{C}_{16:0}$ ).



Oleate (ionized form of oleic acid) ( $\text{C}_{18:1}$ )

Under physiological conditions, these fatty acids are ionized and so they may also be referred to as palmitate and oleate for emphasis on this. In phosphoglycerides, the hydroxyl groups at C-1 and C-2 of the glycerol backbone are esterified to the carboxyl groups of two fatty acid chains while the C-3 hydroxyl group is esterified to phosphoric acid. The resulting compound, called phosphatidate (or diacylglycerol-3-phosphate), is the simplest phosphoglyceride. Only small amounts of phosphatidate are present in membranes but it is a key intermediate in the biosynthesis of the other phosphoglycerides. The absolute configuration of the glycerol-3-phosphate moiety of membrane lipids is shown in the Fig.

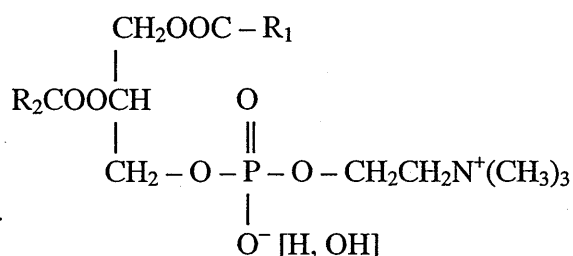
1.3.



**Fig. 1.3** Absolute configuration of the glycerol-3-phosphate moiety of membrane lipids: (A) H and OH, attached to C-2, are in front of the plane of the page, whereas C-1 and C-3 are behind it; (B) Fischer representation of this structure. In a Fischer projection, horizontal bonds denote bonds in front, whereas vertical bonds denote bonds behind the plane of the page.

The major phosphoglycerides are derived from phosphatidate. The phosphate group of phosphatidate is esterified to the hydroxyl group of one of several alcohols. The five common alcohol moieties of phosphoglycerides are ethanolamine, inositol, choline, serine and glycerol.

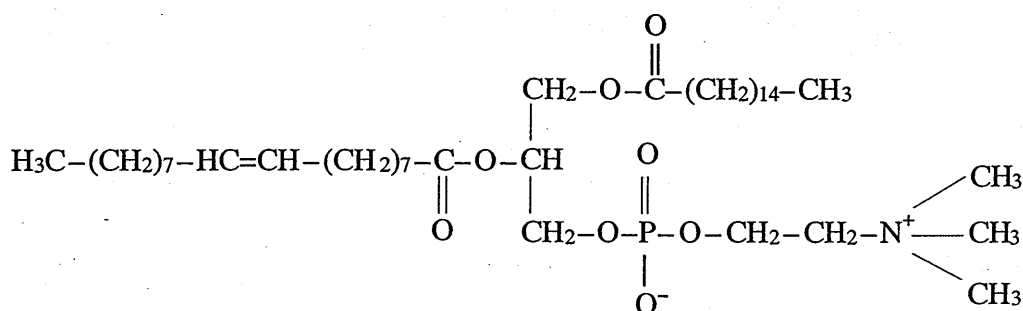
Phosphatidyl choline (PC) (Fig. 1.4) is a phosphoglyceride found in most biological membranes of higher organisms. The word “phosphatidyl” specifically means diacyl glycerophosphoric acid. PC is the most widely distributed phospholipid. In many tissues, PC makes up about half of the total PL fraction. It is a neutral or zwitterionic phospholipid over a pH ranging from strongly acidic to strongly alkaline. In animal tissues, stearic acid and arachidonic acid are commonly esterified to the sn-1 and sn-2 positions (respectively) of PC.



**Fig. 1.4**      Phosphatidylcholine (PC).

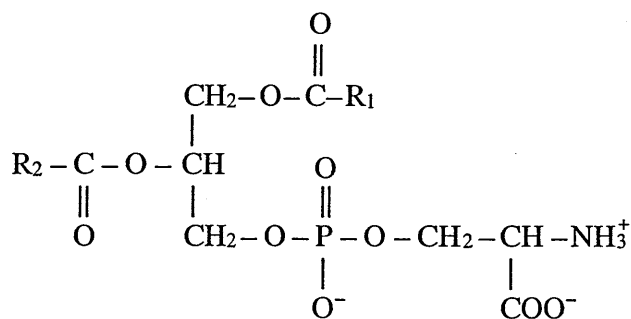
The phospholipid molecule is therefore not one pure compound but a mixture of closely related molecules differing in fatty acid composition. This is a property of all known tissue phospholipids. Enzymatic evidence suggests that the sn-1 position of PC is esterified largely to saturated fatty acids, and the sn-2 position to unsaturated fatty acids (Sugimoto and Yamashita, 1999).





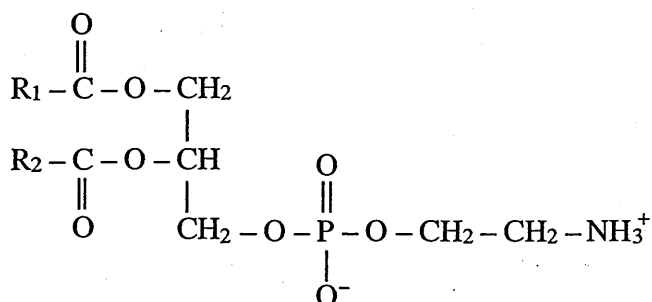
**Fig. 1.5** A phosphatidylcholine (1-palmitoyl-2-oleoyl-phosphatidylcholine).

Phosphatidylserine (PS) is the only amino acid containing glycerophospholipid in animal cells. It is an acidic phospholipid usually occurring in tissues as a salt with  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ . Though PS is widely distributed, it is usually a minor PL component in most cell types. In animal brain cells, AA or DHA is commonly localized at the sn-2 position and stearic acid acylated at the sn-1 position of PS.



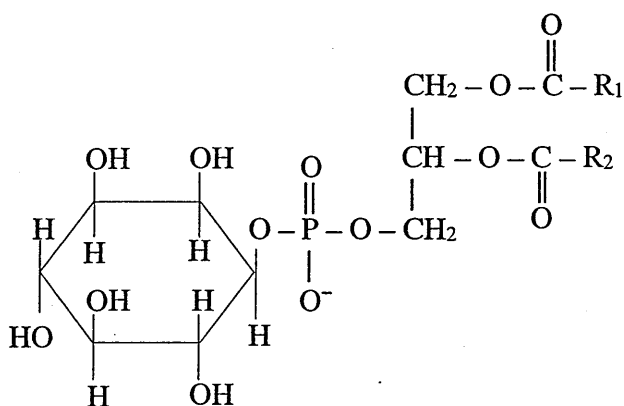
**Fig. 1.6** Phosphatidylserine.

Phosphatidylethanolamine (PE) is a neutral or zwitterionic phospholipid (at least in the pH range 2-7). PE in animal tissues commonly has stearic acid at the sn-1 position and AA at the sn-2 position.



**Fig. 1.7** Phosphatidylethanolamine.

Phosphatidylinositol (PI) is an acidic phospholipid. PI is especially abundant in brain tissues but present in all tissues and cell types. Stearic acid is most commonly acylated at the sn-1 position and AA at the sn-2 position of PI in animal tissues.

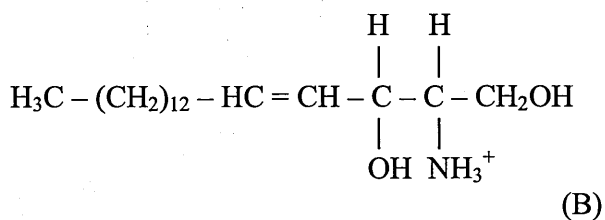
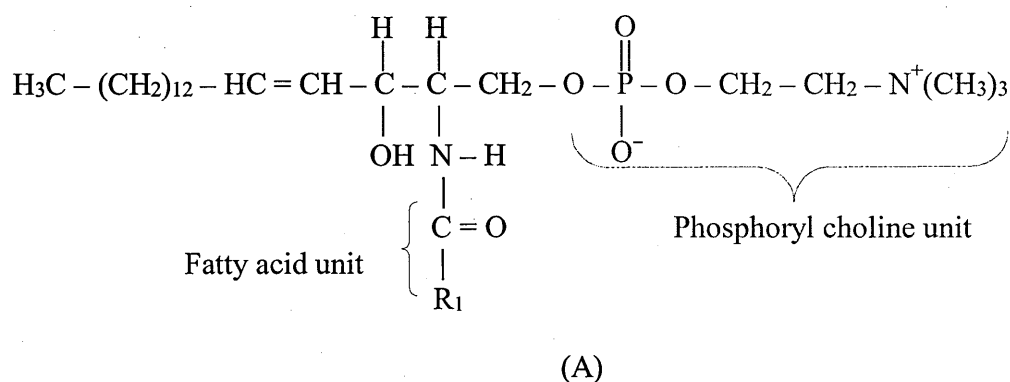


**Fig. 1.8** Phosphatidylinositol.

In general, all double bonds in fatty acids i.e. R<sub>1</sub> and R<sub>2</sub> positions of the glycerol backbone of phospholipids are of the *cis* configuration. A major difference among phospholipids is the charge carried by the polar head groups at neutral pH. Some phosphoglycerides (e.g. PC and PE) have no net electrical charge while others (e.g. PG, cardiolipin (DPG) and PS) have a net negative charge. A few rare phospholipids carry a net positive charge at neutral pH. Nonetheless, the polar head groups of all phospholipids can pack together into the characteristic bi-layer structure.

### 1.8.1.2 Sphingomyelin

As the name implies, sphingomyelin is prominent in myelin sheaths. Sphingomyelin is the only phospholipid without a glycerol backbone. Instead, it has a sphingosine backbone. Sphingosine is an amine alcohol containing a long, unsaturated hydrocarbon chain. In sphingomyelin, the amino group of the sphingosine backbone is linked to a fatty acid by an amide bond. In addition, the primary hydroxyl group of sphingosine is esterified to phosphoryl choline. Thus, the conformation of sphingomyelin resembles that of phosphatidylcholine.



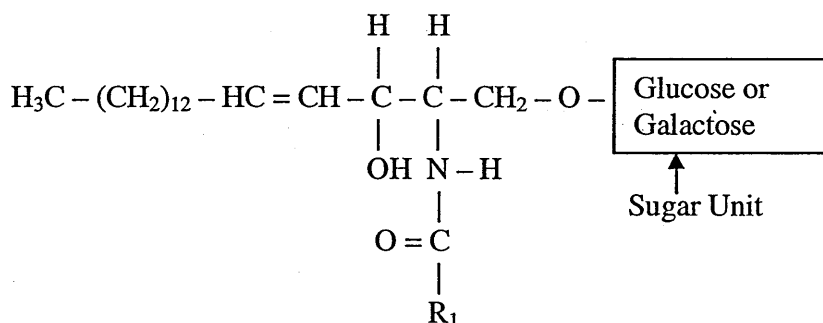
**Fig. 1.9** (A) Sphingomyelin. (B) Sphingosine backbone.

Sphingolipids are important membrane components present in large amounts in the brain and nerve tissues. All sphingolipids contain: one fatty acid molecule, a sphingosine backbone i.e. a sphingosine molecule or one of its derivatives and a polar head group.

### 1.8.2 Glycolipids

As the name implies, glycolipids are sugar-containing lipids. In animal cells, glycolipids like sphingomyelins are derived from sphingosine. The amino group of the sphingosine backbone is acylated by a fatty acid, as in sphingomyelin. Glycolipids however differ from sphingomyelin in the nature of the unit that is linked to the primary hydroxyl group of the sphingosine backbone. In glycolipids, one or more sugars (instead of phosphoryl choline) are attached to this group. The simplest glycolipid is cerebroside, with a single hexose sugar moiety, either glucose or galactose (Fig. 1.10).

Glycolipids are also called glycosphingolipids. Sphingomyelins and glycolipids are similar in shape to phosphoglycerides and can form mixed bi-layers with them. More complex glycolipids, such as gangliosides, contain a branched chain with three or more sugar residues of which at least one is sialic acid, attached to the primary alcohol of sphingosine.

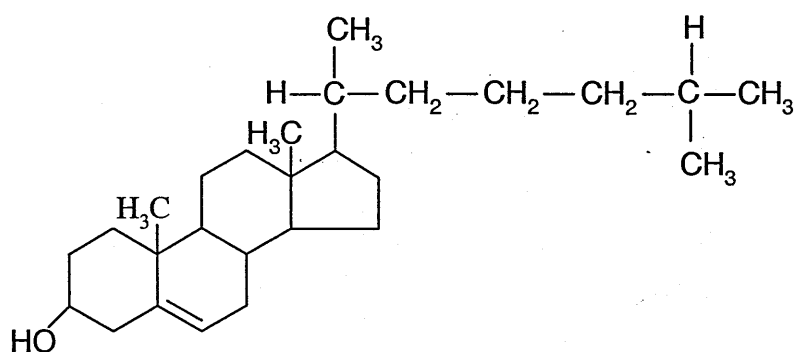


**Fig. 1.10** Cerebroside (a glycolipid).

### 1.8.3 Cholesterol

Cholesterol is an important neutral lipid found in some membranes belonging to the group of sterols. It is the most common sterol in membranes, existing almost exclusively in the plasma membrane of mammalian cells. It can be found in lesser amounts in the membranes

of cellular organelles such as the mitochondria, golgi bodies and nucleus. Cholesterol is generally more abundant towards the outside of the plasma membrane. It intercalates among the phospholipids of the membrane, with its hydroxyl group at the aqueous interface and the remainder of the molecule within the leaflet. This sterol is present in eukaryotes but not in most prokaryotes.



**Fig. 1.11** A cholesterol molecule.

#### 1.8.4 Fatty acid metabolism

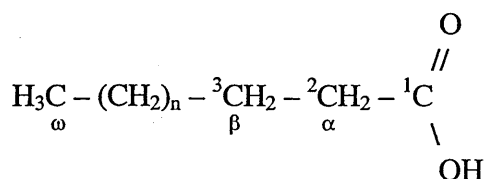
Physiological functions of fatty acids

- Fatty acids are building blocks of phospholipids and glycolipids. They are amphipathic molecules playing critical roles in biological membranes.
- Many proteins are modified by the covalent attachment of fatty acids, which targets them to membrane locations. Soluble cytosolic proteins can be anchored by covalent bonds to a fatty acyl group via myristoylation at the N-terminus or palmitoylation of cysteine residues.
- Fatty acids are fuel molecules stored as triacylglycerols, which are uncharged esters of glycerol (neutral fats).

- Fatty acid derivatives function as hormones and intracellular messengers. For example, prostaglandins, leukotrienes and thromboxanes (local hormones called eicosanoids) derived from arachidonic acid (C<sub>20:4</sub>).

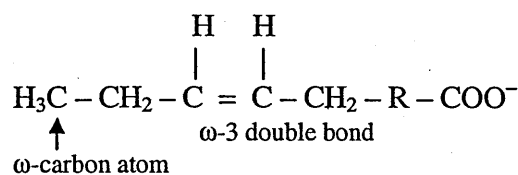
### 1.8.5 Fatty acid Nomenclature

The systemic names of fatty acids are derived from its parent hydrocarbon by the substitution of 'oic' for the last 'e'. For example, C<sub>18</sub> (saturated fatty acid) is called octadecanoic acid as the parent hydrocarbon is octadecane. A C<sub>18</sub> fatty acid with one double bond is called octadecenoic acid; with two double bonds, octadecadienoic acid; and with three double bonds called octadecatrienoic acid. The symbol 16:0 denotes a C<sub>16</sub> fatty acid with no double bonds while 18:2 shows that there are two double bonds. Fatty acid carbon atoms are numbered starting from the carboxy terminus (Fig. 1.12).



**Fig. 1.12** The numbering of fatty acid carbon atoms.

The second and third carbon atoms are usually referred to as  $\alpha$  and  $\beta$ , respectively, whereas the methyl carbon atom at the distal end of the chain is called the  $\omega$ -carbon. The position of a double bond is denoted by the symbol  $\Delta$  followed by a superscript number. For example, cis-  $\Delta^9$  represents a cis double bond between carbon atoms 9 and 10. Trans-  $\Delta^3$  means that there is a trans double bond between carbon atoms 3 and 4. Alternatively, the position of a double bond can be denoted by counting from the distal end with the  $\omega$ -carbon atom (the methyl carbon) as number 1.



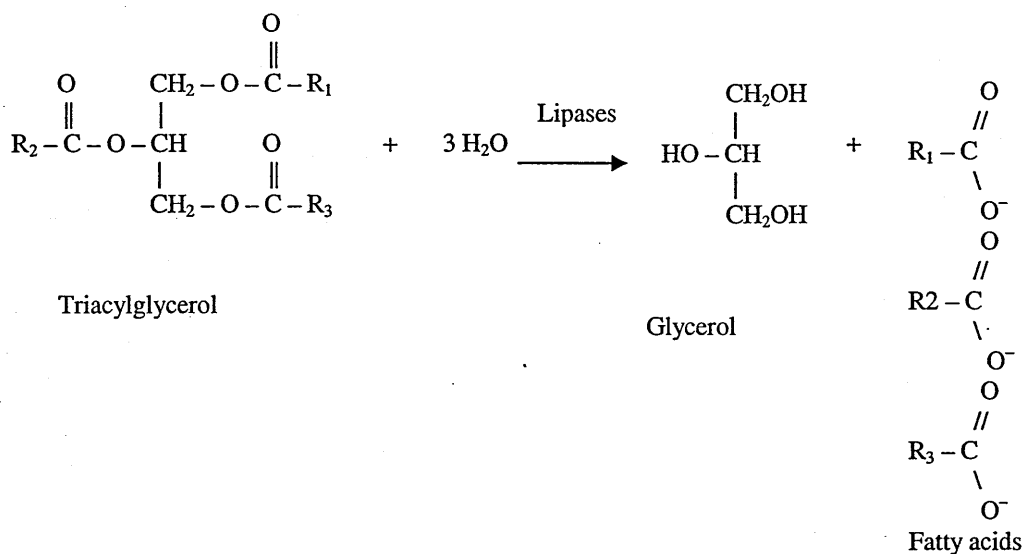
**Fig. 1.13** Structure of an  $\omega$ -3 fatty acid.

Although the systemic naming of fatty acids has been described, the present study refers to them by their trivial names because these names are still more commonly used.

### 1.8.6 Properties of fatty acids

- In biological systems, fatty acids usually contain even numbers of carbon atoms, usually between 12 and 24. In animals, the hydrocarbon chain is almost invariably unbranched. The alkyl chain may be saturated or unsaturated (containing one or more double bonds). The configuration of double bonds in most unsaturated fatty acids is *cis*. The double bonds in PUFAs are separated by at least one methylene group.
- The properties of fatty acids and the lipids derived from them are largely dependent on their chain length and degree of saturation. Saturated fatty acids have a higher melting point than unsaturated fatty acids. For example, stearic acid has a melting point of 69.6°C while oleic acid (containing one *cis* double bond) has a melting point of 13.4°C. The melting point for PUFAs of the C<sub>18</sub> series is even lower. Chain length also affects melting points of fatty acids. The melting point of palmitic acid (C<sub>16</sub>) is 6.5°C lower than that of stearic acid (C<sub>18</sub>) i.e. the shorter the chain length, the lower the melting point. Hence, short chain length and unsaturation enhances the fluidity of fatty acids and their derivatives.

- Triacylglycerols (TAGs) are highly concentrated energy stores because they are reduced and anhydrous. The energy yield from the complete oxidation of fatty acids is about 9kcal/g, in contrast to about 4kcal/g for carbohydrates and proteins. In addition, TAGs are highly non-polar and so stored in nearly anhydrous forms, whereas proteins and carbohydrates are much more polar and as such largely hydrated. In actual fact, 1 g of dry glycogen binds about 2 g of water. Consequently, a gram of nearly anhydrous fat stores more than six times as much energy as a gram of hydrated glycogen. Thus TAGs, rather than glycogen, were selected in evolution as the major energy reservoir.
- TAGs are hydrolysed by lipases (Fig. 1.14).



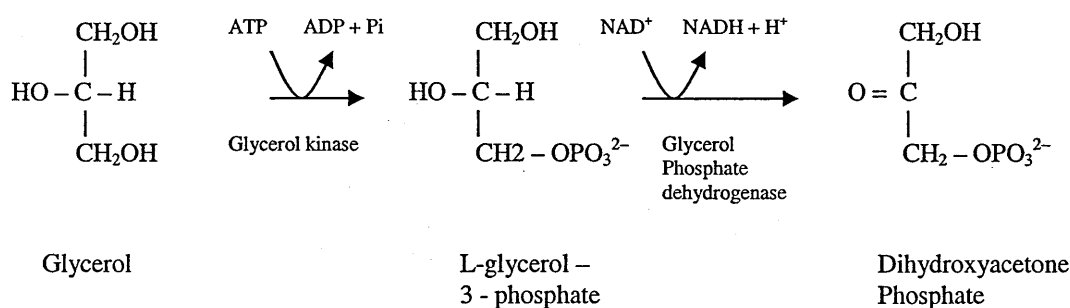
**Fig. 1.14** Hydrolysis of triacylglycerols (TAGs).

The activity of adipose cell lipase is regulated by epinephrine, norepinephrine, glucagon and adenocorticotrophic hormone. These hormones stimulate the adenylate cyclase enzyme of adipose cells, which raises the intracellular concentrations of cyclic adenosine



monophosphate (cAMP). Increased levels of cAMP trigger a protein kinase, which in turn activates the lipase enzyme by phosphorylating it.

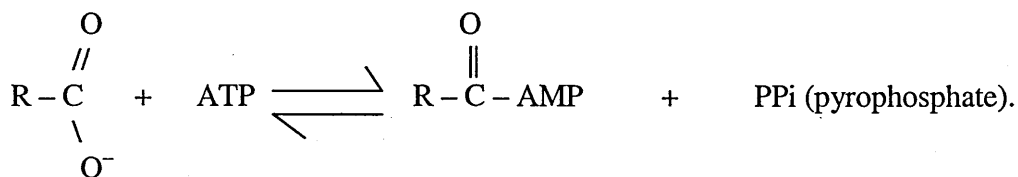
Glycerol formed by lipolysis is phosphorylated and oxidized to dihydroxyacetone phosphate, which is then isomerized to glyceraldehyde-3-phosphate (Fig. 1.15). This intermediate is on both glycolytic and the gluconeogenic pathways. As such, glycerol can be converted into pyruvate or glucose in the liver, which contains the appropriate enzymes. The reverse process can occur by the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, which is hydrolysed by a phosphatase to give glycerol. Thus glycerol and glycolytic intermediates are readily inter-convertible.



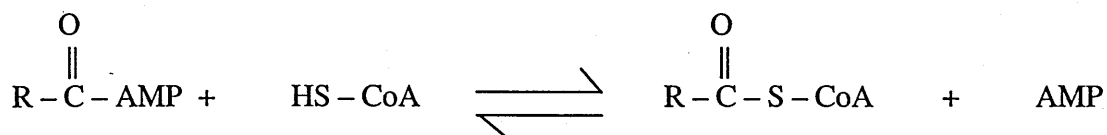
**Fig. 1.15** Phosphorylation of glycerol.

### 1.8.7 Fatty acid breakdown

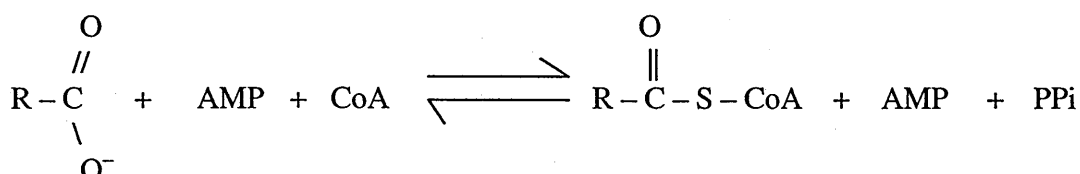
Fatty acids are degraded by oxidation at the  $\beta$  - carbon. Oxidation of fatty acids takes place in the mitochondria. Fatty acid activation occurs in two steps. First, the fatty acid reacts with ATP forming an acyl adenylate:



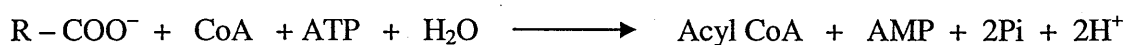
The sulfhydryl group of CoA then attacks the acyl adenylate, which is tightly bound to the enzyme, to form acyl CoA and AMP:



Giving:

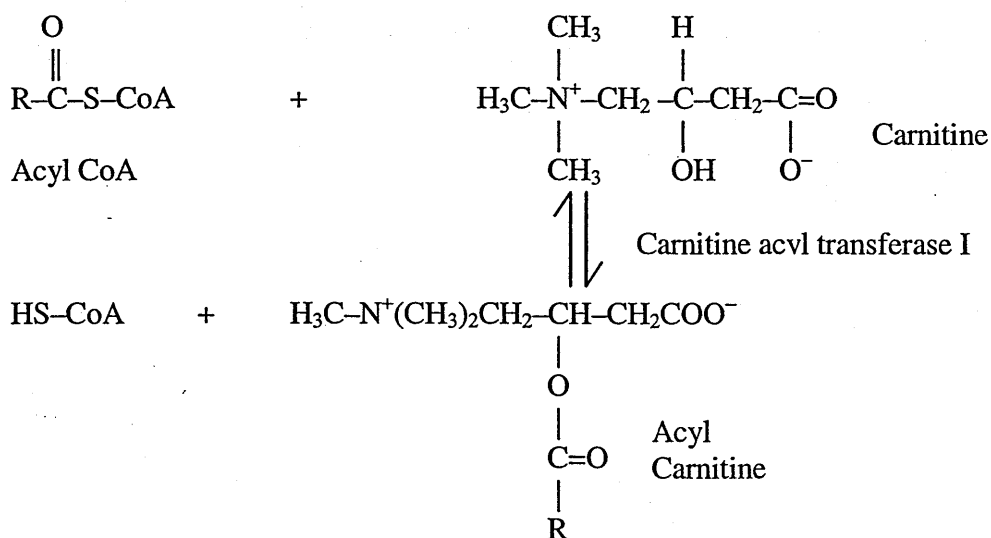


PPi is then rapidly hydrolysed by a pyrophosphatase, making the overall reaction:



The above reaction is irreversible because 2 high-energy bonds are consumed whereas, only one is formed.

Fatty acids are activated on the outer mitochondrial membrane and oxidized in the mitochondrial matrix. Long chain acyl CoA molecules do not readily traverse the inner mitochondrial membrane, hence, a special transport mechanism is required. Activated long chain fatty acids are transported across the inner mitochondrial membrane by carnitine, a zwitterionic compound formed from lysine. The acyl group is transferred from the sulfur atom of CoA to the hydroxyl group of carnitine to form acyl carnitine. This reaction (Fig. 1.16) is catalyzed by carnitine acyl transferase I, located on the cytosolic face of the inner mitochondrial membrane.

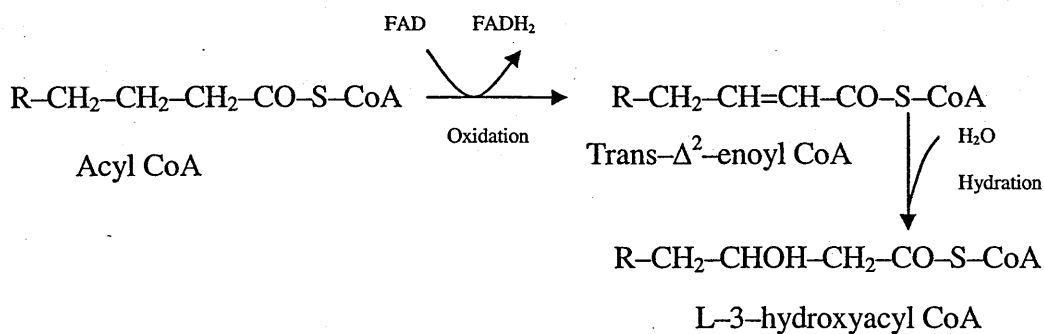


**Fig. 1.16** The transport of long chain acyl CoA molecules across the inner mitochondrial membrane.

Acyl carnitine is then shuttled across the inner mitochondrial membrane by a translocase. The acyl group is transferred back to CoA on the matrix side of the membrane. This reaction is catalyzed by carnitine acyl transferase II. Finally, carnitine is returned to the cytosolic side by the translocase, in exchange for an incoming acyl carnitine. A defect in the transferase or translocase, or a deficiency of carnitine, might impair the oxidation of long-chain fatty acids. Fatty acid oxidation is the major energy yielding process in the following three states: fasting, exercise or a high fat diet. Carnitine is not required for the transfer of medium chain ( $\text{C}_8$  and  $\text{C}_{10}$ ) acyl CoAs into the mitochondrial matrix.

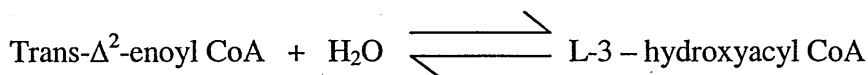
### 1.8.8 $\beta$ -Oxidation pathway of fatty acids

In the  $\beta$ -oxidation pathway of fatty acids, the first reaction is the oxidation of acyl CoA by an acyl CoA dehydrogenase to give an enoyl CoA with a trans double bond between C-2 and C-3 (Fig. 1.17).

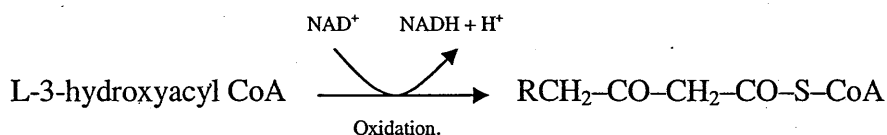


**Fig. 1.17** First reaction of the  $\beta$ -oxidation pathway.

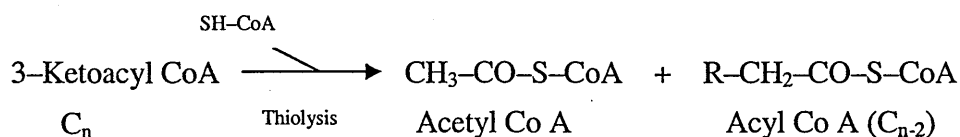
The second reaction is the hydration of the double bonds between C-2 and C-3 by enoyl CoA hydratase.



This is followed by the second oxidation reaction, which converts the hydroxyl group at C-3 into a keto group, generating NADH. It is a reversible reaction.



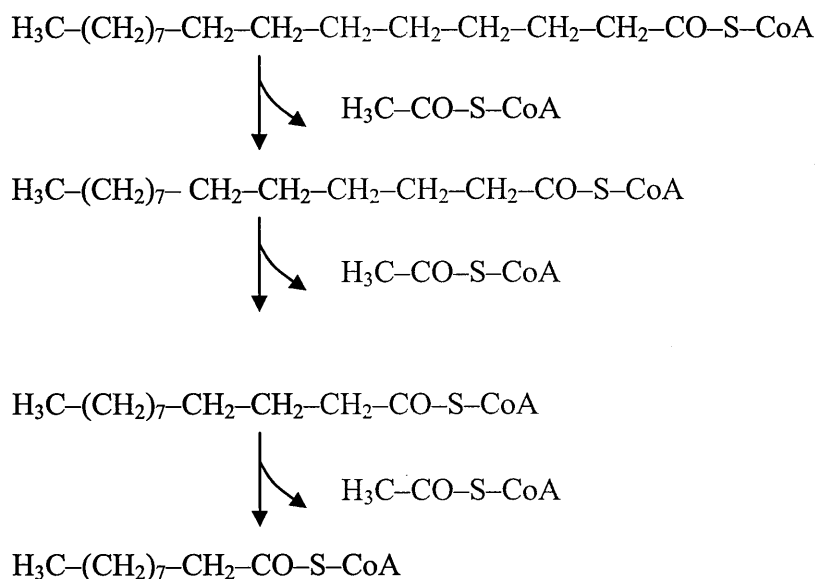
The final step is the cleavage of 3-ketoacyl CoA by the thiol group of a second molecule of CoA, yielding acetyl CoA and an acyl CoA shortened by 2 carbon atoms. This thiolytic cleavage is catalyzed by  $\beta$ -ketothiolase.



The shortened acyl CoA then undergoes another cycle of oxidation, starting with the reaction catalyzed by acyl CoA dehydrogenase.

- fatty acyl chains with 12 to 18 carbons are oxidized by the long chain acyl CoA dehydrogenase.
- The medium-chain acyl CoA dehydrogenase oxidizes fatty acyl chains with 14 to 4 carbons whereas,
- The short-chain acyl CoA dehydrogenase acts on 4- and 6- carbon acyl chains.

In the first three rounds of degradation of palmitate for instance, two carbon units are sequentially removed from the carboxyl end of the fatty acid.

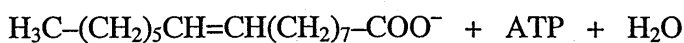


### 1.8.9 Oxidation of unsaturated fatty acids

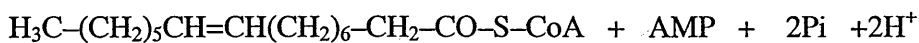
Many of the reactions are similar to those for saturated fatty acids with only two additional enzymes: an isomerase and a reductase needed to degrade a wide range of unsaturated fatty acids. Take the oxidation of palmitoleate for example, this C<sub>16</sub> unsaturated fatty acid with

one double bond between C<sub>9</sub> and C<sub>10</sub>, is activated and transported across the inner mitochondrial membrane in a similar manner as saturated fatty acids.

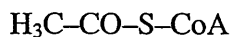
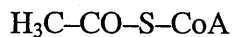
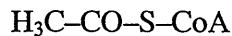
Palmitoleoyl CoA then undergoes three cycles of degradation, which are carried out by the same enzymes as in the oxidation of saturated fatty acids. Three stages of degradation of palmitoleoyl CoA are as follows:



Palmitoleate



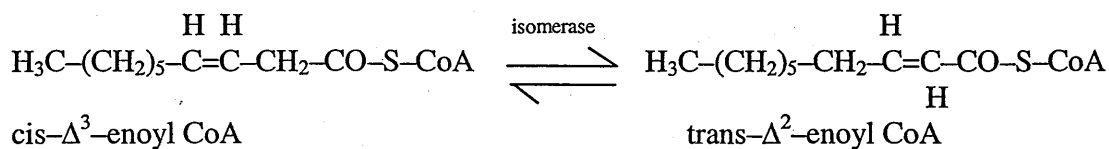
Palmitoleoyl CoA



cis- $\Delta^3$ -enoyl CoA.

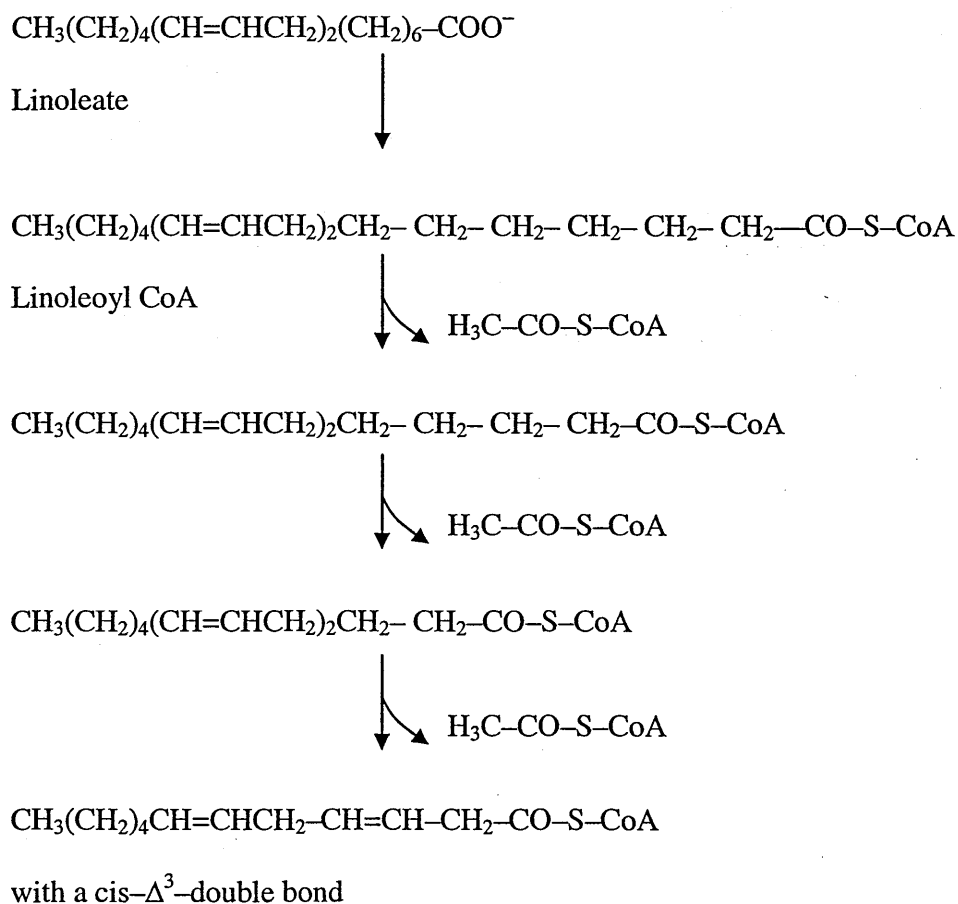
However, the cis- $\Delta^3$ -enoyl CoA formed in the third round is not a substrate for acyl CoA dehydrogenase. The presence of a double bond between C-3 and C-4 inhibits the formation of another double bond between C-2 and C-3. This problem is resolved by a new reaction

that shifts the position and configuration of the cis- $\Delta^3$ -enoyl bond. An isomerase converts this double bond into a trans- $\Delta^2$ -double bond. The subsequent reactions are those of the saturated fatty acid oxidation pathway, in which the trans- $\Delta^2$ -enoyl CoA is a regular substrate.

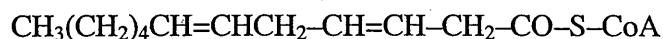


### 1.8.10 Oxidation of polyunsaturated fatty acids

Consider linoleate, a  $\text{C}_{18}$  PUFA with cis- $\Delta^9$  and cis- $\Delta^{12}$  double bonds, after 3 rounds of  $\beta$ -oxidation:



The cis- $\Delta^3$  double bond formed after 3 rounds of  $\beta$  - oxidation is converted by the isomerase into a trans- $\Delta^2$  double bond. However, the cis- $\Delta^{12}$  double bond of linoleate poses a new problem. After 3 rounds of  $\beta$  - oxidation of linoleate, the following reaction takes place:

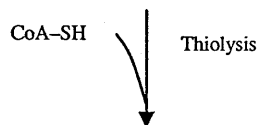
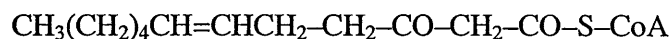
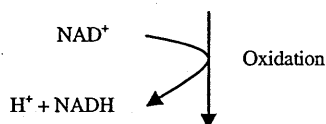
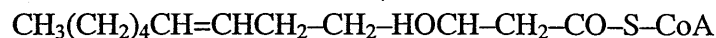
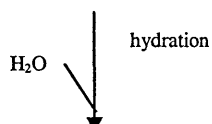


cis, cis- $\Delta^3, \Delta^6$ -dienoyl CoA

isomerase



2, 6-dienoyl CoA.

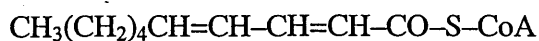
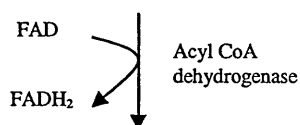


Acyl CoA

Acetyl CoA

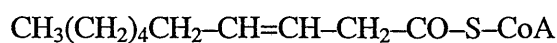
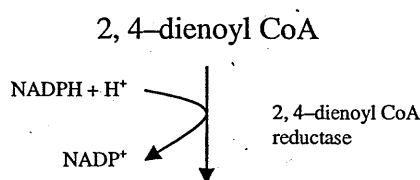
The acyl CoA above is produced by 4 rounds of  $\beta$  - oxidation. It contains a cis- $\Delta^4$  double bond. Dehydrogenation of this species by acyl CoA dehydrogenase yields a 2, 4 - dienoyl intermediate, which is not a substrate for the next enzyme in the  $\beta$  - oxidation pathway.



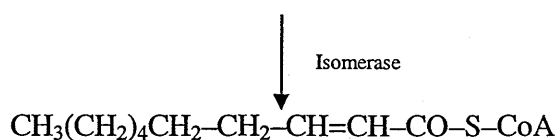


Trans  $\Delta^2$ , cis  $\Delta^4$ -dienoyl CoA (2, 4-dienoyl CoA)

The cis  $\Delta^4$  problem from the above product is overcome by 2, 4-dienoyl CoA reductase, an enzyme utilizing NADPH to reduce the 2, 4-dienoyl intermediate to cis  $\Delta^3$ -enoyl CoA.



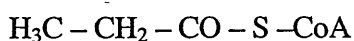
cis  $\Delta^3$ -enoyl CoA



trans  $\Delta^2$ -enoyl CoA

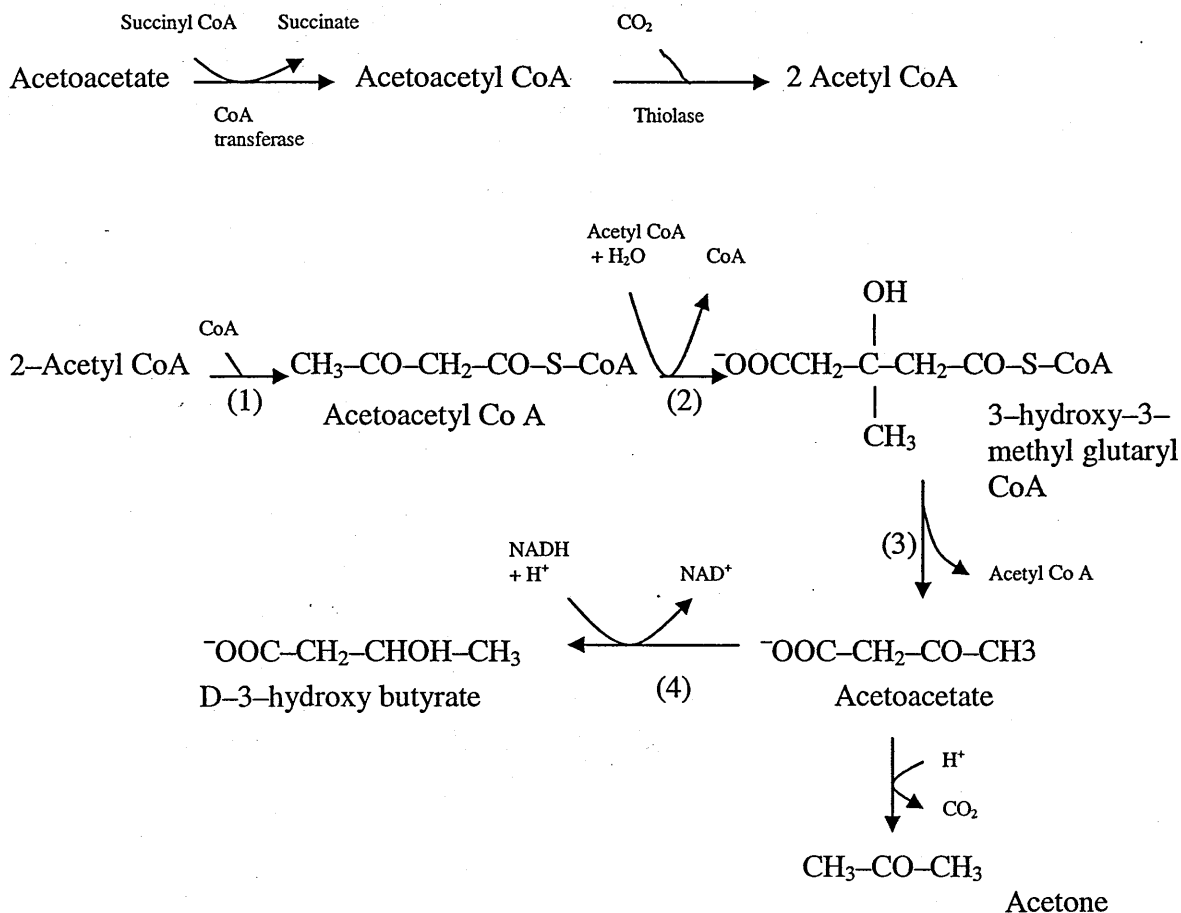
Only two extra enzymes are required for the  $\beta$ -oxidation of any PUFA. Odd numbered double bonds are handled by the isomerase, and even-numbered ones by the reductase and the isomerase. A round of  $\beta$  - oxidation of saturated fatty acids includes: oxidation by FAD (flavin adenine dinucleotide); hydration; oxidation by  $\text{NAD}^+$  and thiolysis by CoA. Odd-numbered fatty acids are minor species, oxidized in the same fashion as even-numbered ones except that propionyl CoA and acetyl CoA, rather than two molecules of acetyl CoA,

are produced in the final round of degradation. The activated 3-C unit of propionyl CoA enters the Krebs cycle after conversion into succinyl CoA.



Propionyl CoA

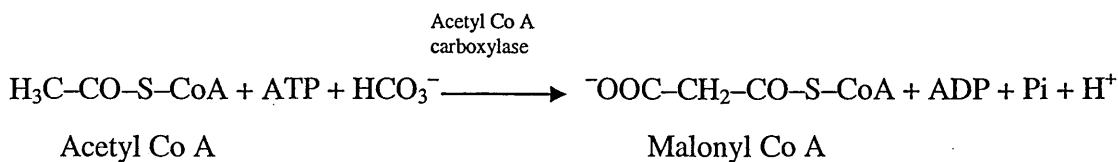
The acetyl CoA formed from fatty acid oxidation enters the Krebs cycle only if fat and carbohydrate degradation are appropriately balanced. The reason being that the entry of acetyl CoA into the Krebs cycle is dependent on the availability of oxaloacetate for the formation of citrate, but the concentration of oxaloacetate is lowered if carbohydrate is unavailable or improperly used. Oxaloacetate is normally produced from pyruvate, generated from glycolysis. In fasting or diabetes, oxaloacetate is converted to glucose by the gluconeogenic pathway making it unavailable for condensation with acetyl CoA. Under such conditions, acetyl CoA is converted to acetoacetate and D-3-hydroxybutyrate. Acetoacetate, D-3-hydroxybutyrate and acetone are sometimes referred to as ketone bodies. The major site for the production of acetoacetate and 3-hydroxybutyrate is the liver. They are both normal fuels of respiration and are quantitatively important as sources of energy. Glucose is the major fuel for the brain and red blood cells in well-nourished people on a balanced diet. However, the brain utilizes acetoacetate during starvation and diabetes. In prolonged starvation, 75% of the fuel needs of the brain are met by acetoacetate (a water-soluble, transportable form of acetyl units). Acetoacetate can be activated by the transfer of CoA from succinyl CoA in a reaction catalyzed by a specific CoA transferase. Acetoacetyl CoA is then cleaved by thiolase to yield 2 molecules of acetyl CoA (Fig. 1.18), which can then enter the Krebs cycle. Animals cannot convert fatty acids into glucose but plants can.



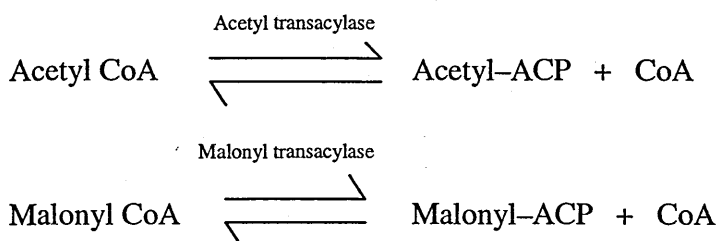
**Fig. 1.18** Activation of acetoacetate where (1): 3-ketothiolase; (2): hydroxymethyl glutaryl CoA synthase; (3): hydroxymethyl glutaryl CoA cleavage enzyme and (4): D-3 hydroxybutyrate dehydrogenase.

### 1.8.11 Biosynthesis of fatty acids

Fatty acids are synthesized and broken down by different pathways and the synthesis is not simply the reversal of the degradative process. Fatty acid synthesis starts by the carboxylation of acetyl CoA to malonyl CoA. This is an irreversible reaction.

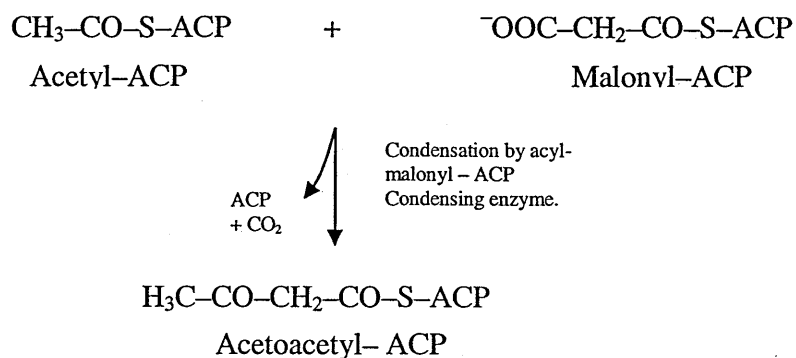


The enzyme system catalyzing the synthesis of saturated long-chain fatty acids from acetyl CoA, malonyl CoA, and NADPH is called fatty acid synthase. The elongation process starts with the formation of acetyl-ACP and malonyl-ACP (acyl carrier protein).

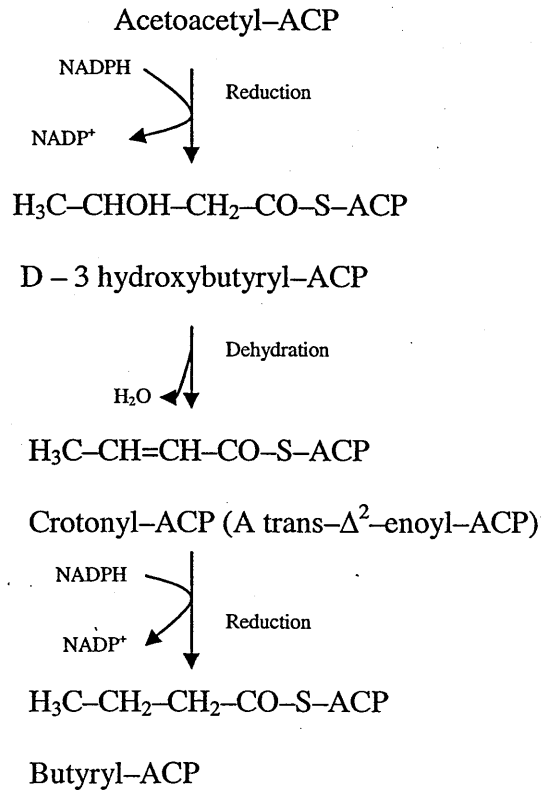


Odd numbered fatty acids are synthesized starting with propionyl-ACP, which is formed from propionyl CoA by acetyl transacylase. Malonyl transacylase is highly specific for its substrate while acetyl transacylase is not.

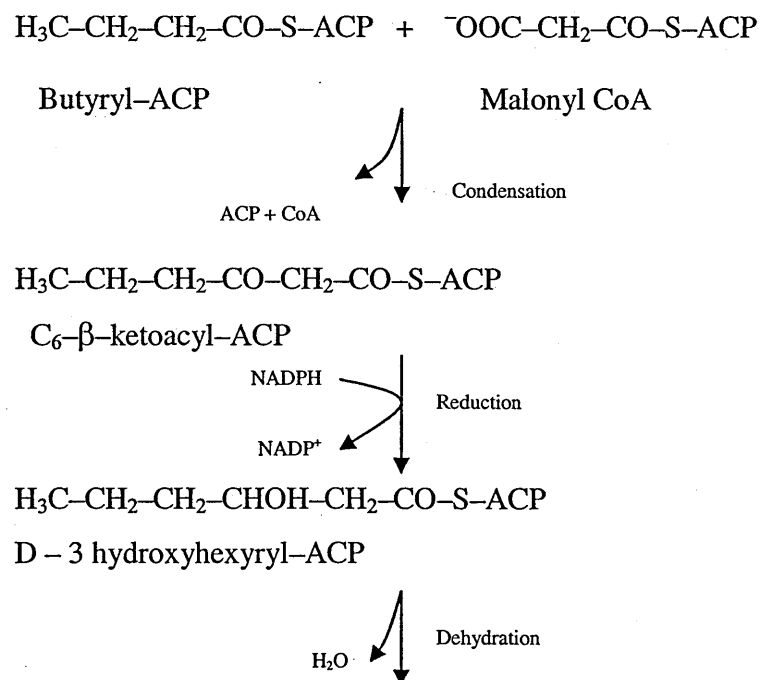
Acetyl-ACP and malonyl-ACP react to form acetoacetyl-ACP.



The next three steps reduce the keto group at C-3 to a methylene group.

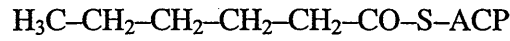
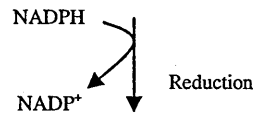


This completes the first elongation cycle. In the second round of fatty acid synthesis, butyryl-ACP condenses with malonyl-ACP to form a C6- $\beta$ -ketoacyl-ACP.



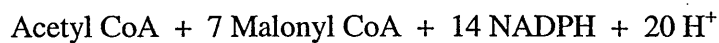


A trans- $\Delta^2$ -enoyl-ACP

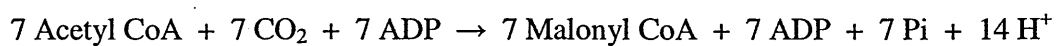


C<sub>6</sub>-Acyl-ACP

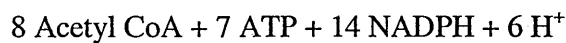
The C-6 acyl ACP, is ready for a third round of elongation. This elongation cycle continues until the formation of C<sub>16</sub>-acyl-ACP, an intermediate that is not a substrate for the condensing enzyme. Instead, it is hydrolyzed to give palmitate and ACP. For the synthesis of palmitate:



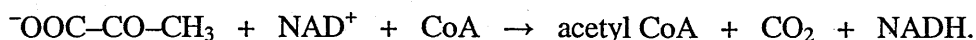
For the synthesis of malonyl CoA used in the above equation:



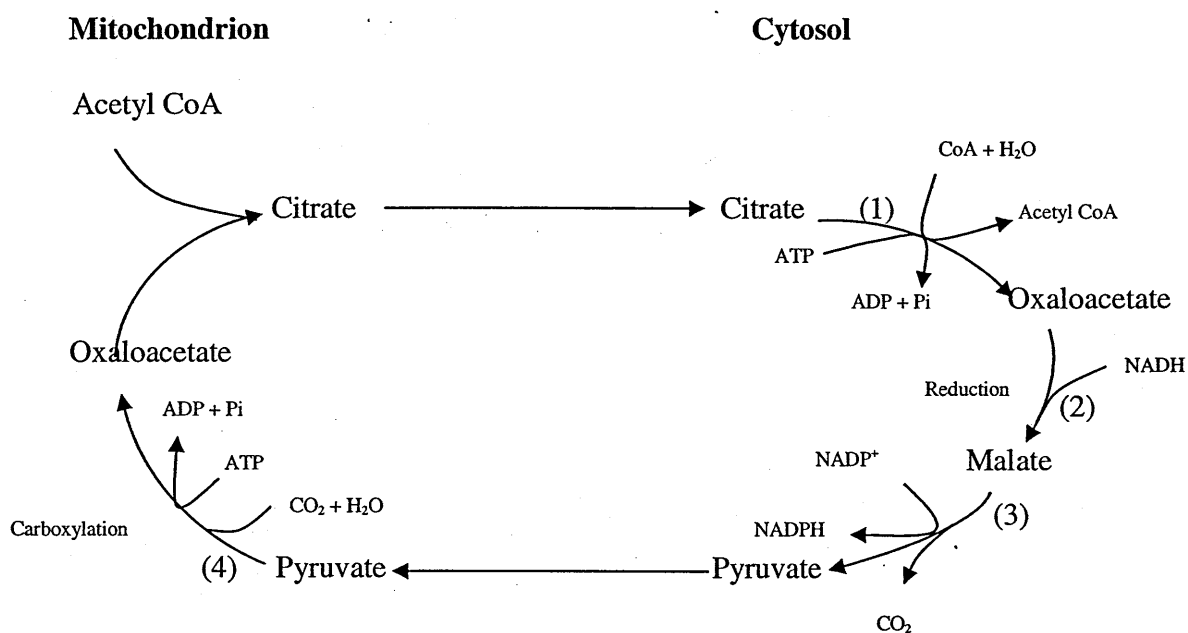
Hence the overall stoichiometric reaction for the synthesis of palmitate is:



Fatty acids are synthesized in the cytosol whereas, acetyl CoA is formed from pyruvate in mitochondria by oxidative decarboxylation:



Acetyl CoA produced in the mitochondria must be transferred to the cytosol for fatty acid synthesis (Fig. 1.19). Mitochondrial membranes are not readily permeable to acetyl CoA, so carnitine carries long-chain fatty acids. The membrane barrier to acetyl CoA is bypassed by citrate, which carries acetyl groups across the inner mitochondrial membrane. Citrate is formed in the mitochondrial matrix by the condensation of acetyl CoA with oxaloacetate.

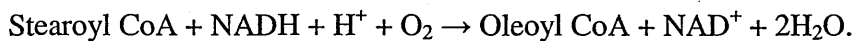


**Fig. 1.19** Transport of acetyl CoA into the cytosol, where (1): Cleavage by citrate lyase; (2): Malate dehydrogenase; (3): Oxidative decarboxylation and (4): Pyruvate carboxylase.

Oxaloacetate formed in the transfer of acetyl groups to the cytosol must now be returned to the mitochondria. Fatty acid metabolism is stringently controlled so that synthetic and

breakdown processes are highly responsive to physiological needs. Fatty acid synthesis and degradation are reciprocally regulated so that both are not simultaneously active.

The major product of fatty acid synthase is palmitate. In eukaryotes, longer fatty acids are formed by elongation reactions catalyzed by enzymes on the cytosolic side of the membranes of the endoplasmic reticulum. To study these reactions, the membrane is fragmented into closed vesicles called microsomes. Microsomal enzymes add 2-carbon units sequentially to the carboxyl end of both saturated and unsaturated fatty acids. Malonyl CoA is the 2-C donor in the elongation of fatty acyl CoAs. Again, condensation is driven by the decarboxylation of malonyl CoA. Microsomal systems also introduce double bonds into long-chain acyl CoAs. For example, in the conversion of stearoyl CoA into oleoyl CoA, a *cis*  $\Delta^9$  double bond is inserted by an oxidase that employs molecular oxygen and NADH (or NADPH).



This reaction is catalyzed by a complex of 3-membrane-bound enzymes: NADH-cytochrome  $b_5$  reductase; cytochrome  $b_5$  and a  $\Delta^9$  desaturase. A variety of unsaturated fatty acids can be formed from oleate by a combination of elongation and desaturation reactions. For example, oleate (18:1 *cis*- $\Delta^9$  fatty acid) can be elongated to a 20:1 *cis*- $\Delta^{11}$  fatty acid. Alternatively, a second double bond can be inserted to yield an 18:2 *cis*- $\Delta^6$ ,  $\Delta^9$  fatty acid. Unsaturated fatty acids in mammals are derived from palmitoleate (16:1n-7), oleate (18:1n-9), linoleate (18:2n-6) or linolenate (18:3n-3). The number of carbon atoms from the  $\omega$ -end of a derived unsaturated fatty acid to the nearest double bond identifies the precursor.

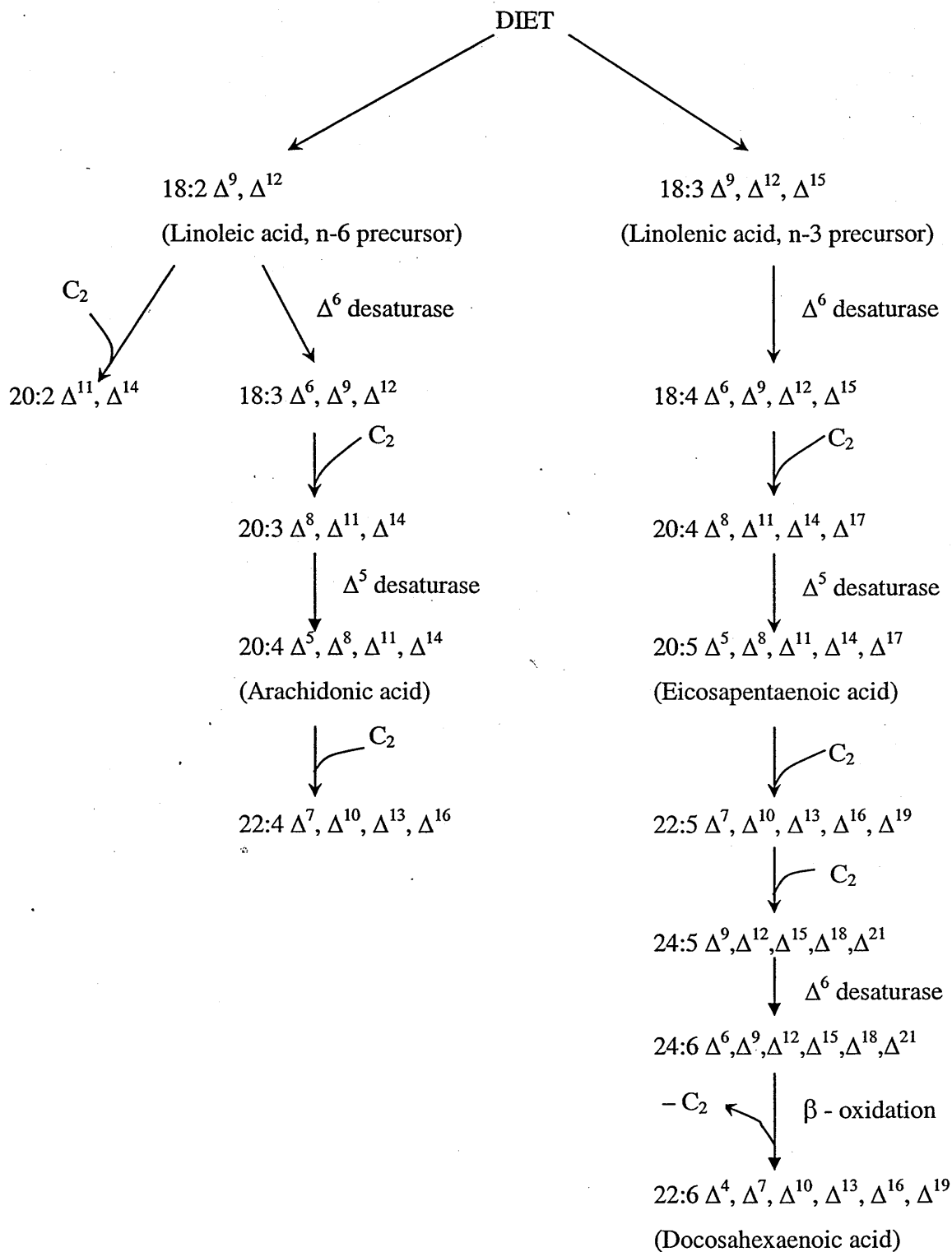


Since  $\Delta^{12}$  and  $\Delta^{15}$  desaturase enzymes do not exist in vertebrates, linoleic and linolenic acids are essential fatty acids.

**Table 1.3** Precursors of unsaturated fatty acids.

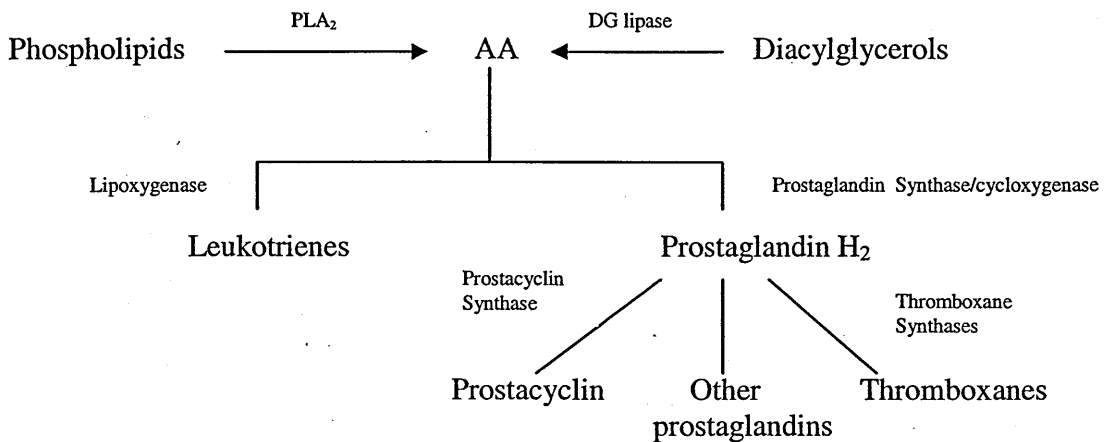
Precursor	Formula
Linolenate (n-3)	$\text{CH}_3\text{--CH}_2\text{--CH=CH--R}$
Linoleate (n -6)	$\text{CH}_3\text{--(CH}_2)_4\text{--CH=CH--R}$
Palmitoleate (n -7)	$\text{CH}_3\text{--(CH}_2)_5\text{--CH=CH--R}$
Oleate (n -9)	$\text{CH}_3\text{--(CH}_2)_7\text{--CH=CH--R}$

Mammals lack the enzymes to introduce double bonds at carbon atoms beyond C-9 in the fatty acid chain. Hence, mammals cannot synthesize linoleate (18:2 cis  $\Delta^9$ ,  $\Delta^{12}$ ) and linolenate (18:3 cis  $\Delta^9$ ,  $\Delta^{12}$ ,  $\Delta^{15}$ ). Linoleate and linolenate are the two dietary essential fatty acids. The term essential means that they must be supplied in the diet because they are required by the organism and cannot be endogenously synthesized. Linoleate and linolenate supplied by the diet are the starting points for the synthesis of the n-6 (arachidonic acid) and n-3 (eicosapentaenoic acid and docosahexaenoic acid) PUFAs, respectively (Fig. 1.20).



**Fig. 1.20** Pathways for the biosynthesis of n-6 and n-3 fatty acids by desaturation and chain elongation in animal tissues.

Arachidonate, a 20:4 cis  $\Delta^5, \Delta^8, \Delta^{11}, \Delta^{14}$  fatty acid is a major precursor of several classes of signal molecules namely, prostaglandins, prostacyclins, thromboxanes and leukotrienes (Fig. 1.21).



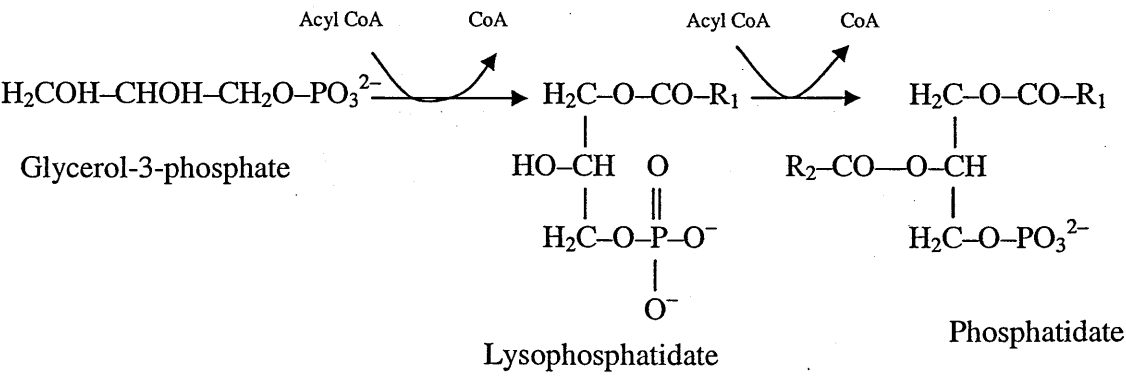
**Fig. 1.21** Breakdown of phospholipids.

Also, eicosapentaenoic acid (EPA), a 20:5 cis  $\Delta^5, \Delta^8, \Delta^{11}, \Delta^{14}, \Delta^{17}$  fatty acid is a precursor for eicosanoids. On the other hand, docosahexaenoic acid (DHA), a 22: 6 cis  $\Delta^4, \Delta^7, \Delta^{10}, \Delta^{13}, \Delta^{16}, \Delta^{19}$  fatty acid is not a substrate for the production of eicosanoids. The major PG classes are denoted PGA through PGI; a subscript denotes the number of C–C double bonds outside the ring. PGs with 2 double bonds such as PGE<sub>2</sub>, are derived from arachidonic acid; the other 2 double bonds of AA (the precursor) are lost in forming a five-member ring. Prostacyclin and Thromboxanes (TX) are related compounds arising from a nascent PG. Alternatively, AA can be converted to leukotrienes by the action of the lipoxygenase. These compounds, were first found in leukocytes, containing three conjugated double bonds – hence the name. Prostaglandins, prostacyclins, leukotrienes and thromboxanes are called eicosanoids because they contain 20 carbon atoms. They are all local hormones since they are short-lived. They alter the activities of the cells in which they are synthesized and of adjoining cells. Contrary to the activities of global hormones

such as insulin and glucagon, the nature of the effect of eicosanoids may vary from one cell type to another. Prostaglandins stimulate inflammation, regulate blood flow to particular organs, control ion transport across membranes, modulate synaptic transmission and affect sleep patterns/rhythms.

### 1.8.12 Biosynthesis of phospholipids

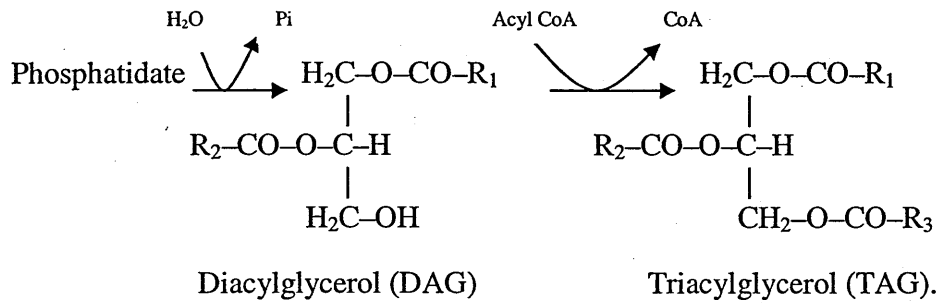
Phosphatidate (diacylglycerol-3-phosphate) is a common intermediate in the synthesis of phosphoglycerols and TAGs. The pathway starts with glycerol-3-phosphate, formed primarily by the reduction of dihydroxyacetone phosphate and to a lesser extent by the phosphorylation of glycerol. Glycerol-3-phosphate is acylated by acyl CoA yielding lysophosphatidate, which is again acylated by acyl CoA to form phosphatidate (Fig. 1.22). These acylations are catalyzed by glycerol phosphate acyl transferase. In most phosphatidates, the fatty acyl chain attached to C<sub>1</sub> is saturated, whilst the one attached to C<sub>2</sub> is unsaturated.



**Fig. 1.22** Acylation of glycerol-3-phosphate.

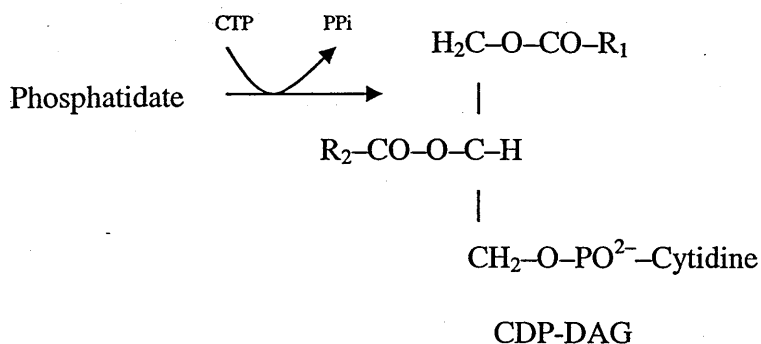
The pathways for the synthesis of TAGs and phosphoglycerides diverge at phosphatidate. In the synthesis of TAGs, phosphatidate is hydrolyzed by a specific phosphatase forming a

diacylglycerol (DAG), which is then acylated to a TAG, in a reaction catalyzed by diglyceride acyl transferase (Fig. 1.23). These enzymes are associated in a TAG synthase complex bound to the endoplasmic reticulum membrane.



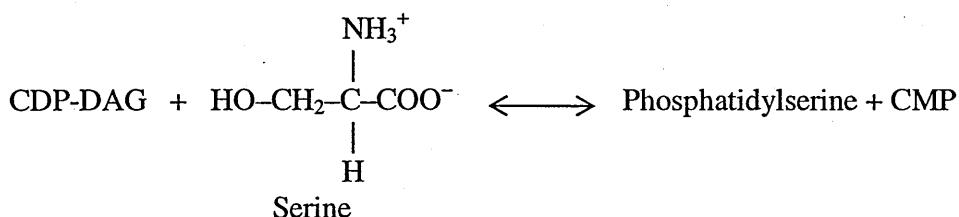
**Fig. 1.23** Synthesis of TAG.

In the synthesis of phosphoglycerides, several pathways can be followed. One *de novo* synthetic pathway begins with the formation of cytidine diphosphodiacyl glycerol (CDP-DAG) from phosphatidate and cytidine triphosphate (CTP) (Fig. 1.24). This reaction is driven forward by the hydrolysis of PPi.



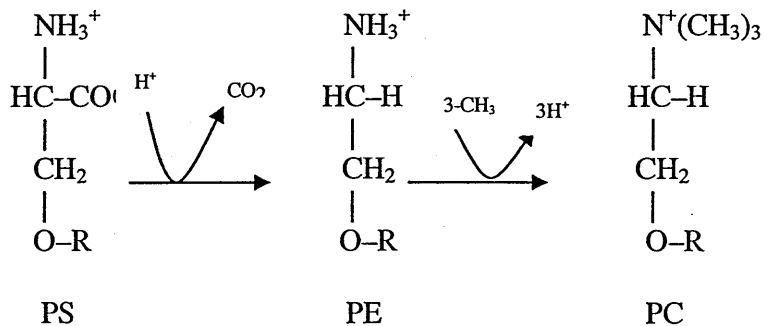
**Fig. 1.24** Synthesis of cytidine diphosphodiacyl glycerol (CDP-DAG).

The activated phosphatidyl unit, reacts with the hydroxyl group of a polar alcohol. If the alcohol is serine, it yields PS and cytidine monophosphate (CMP) (Fig. 1.25).



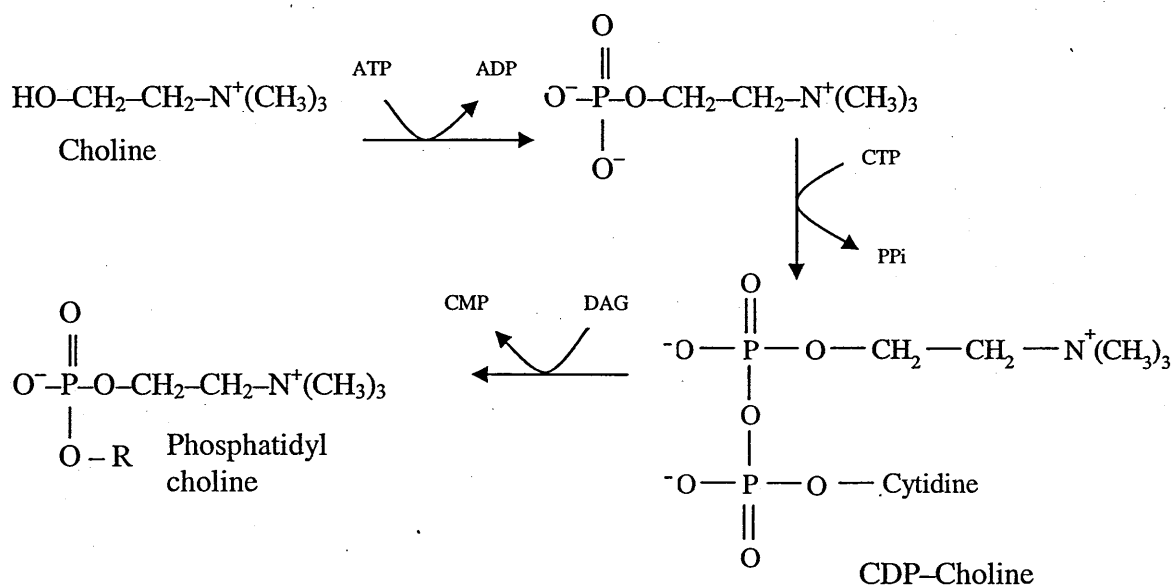
**Fig. 1.25** Synthesis of phosphatidylserine (PS).

In the same way, PI is formed by the transfer of a DAG phosphate unit from CDP-DAG to inositol. PE and PC can be formed from PS (Fig. 1.26). In bacteria, decarboxylation of PS by a pyridoxal phosphate enzyme yields PE.



**Fig. 1.26** Synthesis of phosphatidylethanolamine and phosphatidylcholine.

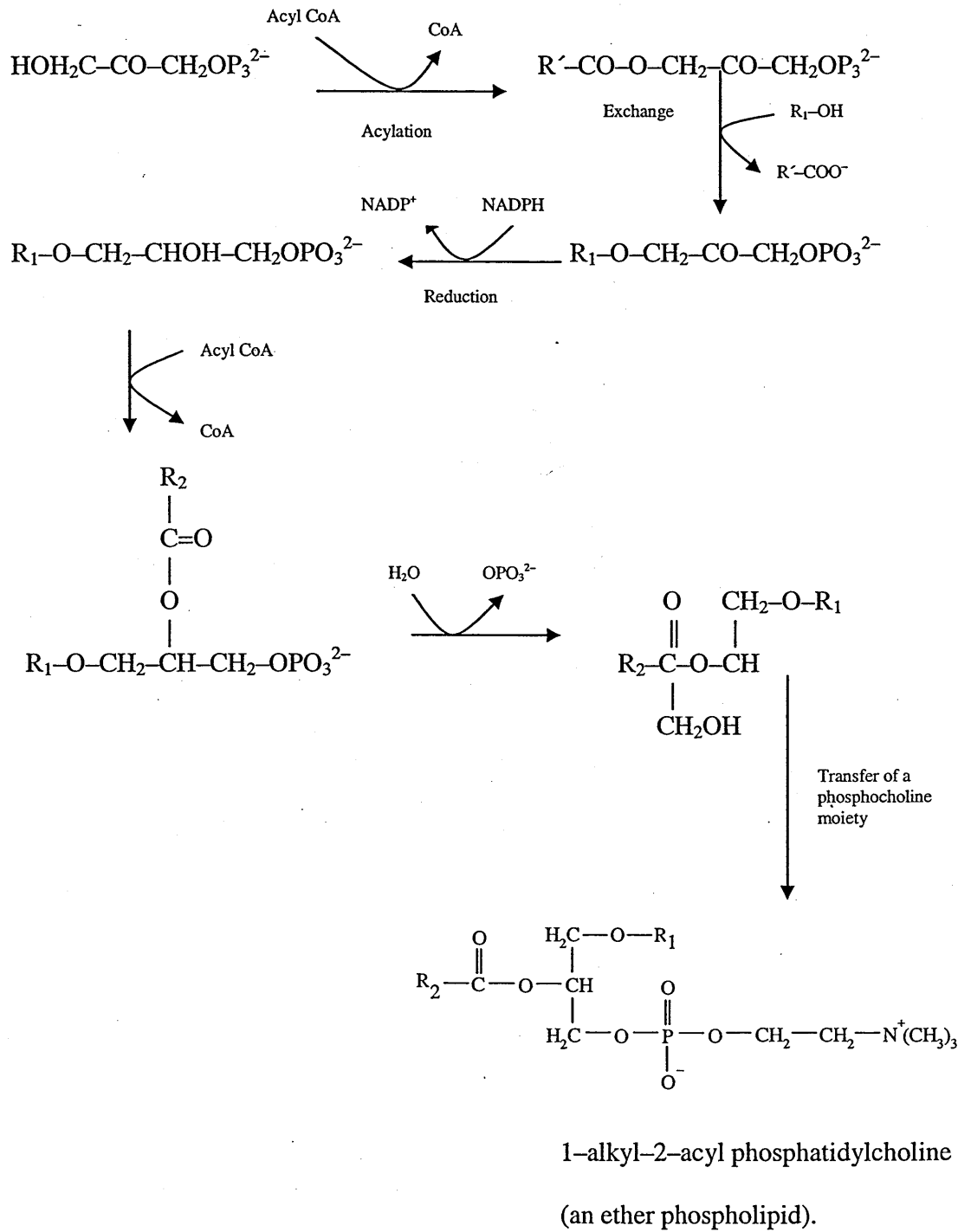
Phosphoglycerides can also be synthesized from a CDP-alcohol intermediate. In mammalian cells, PC is synthesized via a pathway utilizing choline supplied by the diet. Choline is phosphorylated by ATP to phosphoryl choline, which then reacts with CTP forming CDP-choline. The phosphoryl choline unit of CDP-choline is then transferred to a DAG to form phosphatidylcholine (Fig. 1.27). Similarly, PE can be synthesized from ethanolamine by forming CDP-ethanolamine intermediate by analogous reactions. On the other hand, PE can be formed from PS by the enzyme-catalyzed exchange of ethanolamine for the serine moiety of the phospholipid.



**Fig. 1.27** Synthesis of PC.

Some phospholipids possess an ether unit instead of an acyl unit at  $\text{C}_1$ . Glyceryl ether phospholipids are synthesized starting with dihydroxyacetone phosphate. Acylation by a fatty acyl CoA yields a 1-acyl derivative that exchanges with a long-chain alcohol forming an ether at  $\text{C}_1$ . The keto-group at  $\text{C}_2$  is reduced by NADPH, to form an alcohol. The resulting alcohol is acylated by a long-chain CoA. Removal of the 3-phosphate group

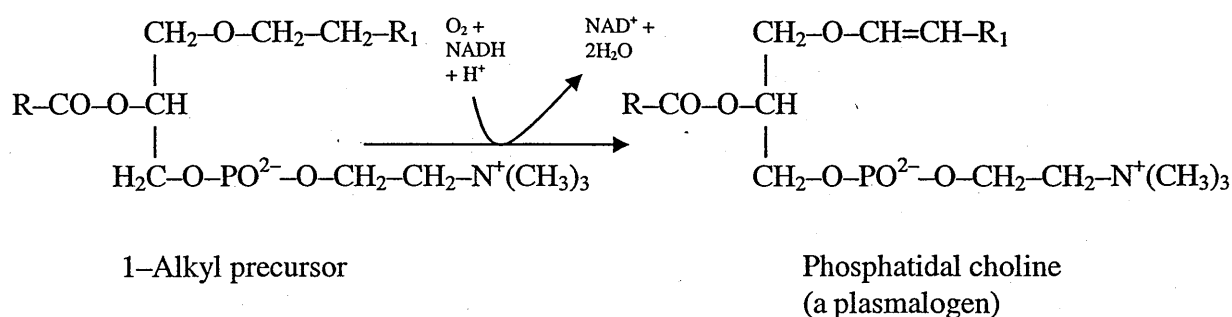
yields 1-alkyl-2-acylglycerol, which then reacts with CDP-choline yielding the other analog of PC (Fig. 1.28).



**Fig. 1.28**      Synthesis of an ether phospholipid.



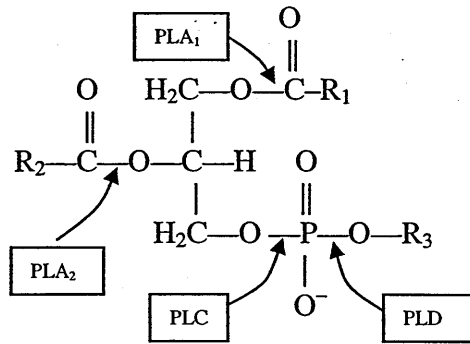
Platelet activating factor (PAF) is an ether phospholipid with striking activity. PAF induces blood platelet aggregation and dilation of blood vessels. The presence of an acetyl group at C<sub>2</sub> increases the solubility of this lipid in the aqueous environment of membranes. Plasmalogens are phospholipids containing  $\alpha$ ,  $\beta$ -unsaturated ether at C<sub>1</sub>. Phosphatidal choline, the corresponding plasmalogen to PC, is formed by the desaturation of a 1-alkyl precursor (Fig. 1.29).



**Fig. 1.29**      Synthesis of phosphatidal choline.

### 1.8.13 Breakdown of phospholipids

Phospholipids are broken down by a group of phospholipases. Phospholipase enzymes preferentially hydrolyze substrates located in the membrane bilayers, micelles or lipoprotein particles, acting on the interfacial location at the boundary of water and a condensed lipid phase. There are four different groups of phospholipases classified according to their specificities namely: phospholipases A<sub>1</sub>, A<sub>2</sub>, C and D. The bonds hydrolyzed by these enzymes are shown in Fig. 1.30.



**Fig. 1.30** Specificity of phospholipases.

Phospholipase A<sub>1</sub> (PLA<sub>1</sub>) hydrolyses the acyl group from the sn-1 position of glycerophospholipids generating FFAs (usually saturated fatty acids) and LPLs. Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) hydrolyses the ester bond of glycerophospholipids at the sn-2 position generating FFAs (usually unsaturated fatty acids) and LPLs. Phospholipase C (PLC) hydrolyses the phosphodiester bond linking the phosphorylated alcohol unit to the acylated glycerol moiety. Phospholipase D (PLD) hydrolyses the ester bond linking the alcohol unit to the phosphorylated glycerol moiety.

Of the four different phospholipase enzymes, PLA<sub>2</sub> will be focused upon in this review because of its implications in the neuropathophysiology of schizophrenia.

#### 1.8.14 Phospholipase A<sub>2</sub> (PLA<sub>2</sub>)

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>; EC 3.1.1.4) enzymes catalyze the breakdown of phospholipids yielding free fatty acids (FFAs) and lysophospholipids (LPLs). They have two major roles. Many of them serve as digestive enzymes present in high concentration in intestinal juices,

bacterial secretions and venoms. They generate highly active signal molecules or their immediate precursors (Fig 1.21).

PLA<sub>2</sub> lipolytic enzymes are widely distributed in nature. They are well-characterized proteins with respect to their catalytic and pharmacological activities. PLA<sub>2</sub>s specifically hydrolyze the sn-2 ester bond of phospholipids (van Deenen and de Haas, 1963), displaying enhanced activity towards lipids in lamellar and micellar aggregates both in membranes and at other lipid - water interphases (Ramirez and Jain 1991; Jain et al., 1995). These enzymes have been classified into 11 groups (I – XI) occurring as extra-cellular and intra-cellular PLA<sub>2</sub>s (Six and Dennis, 2000).

Extracellular PLA<sub>2</sub>s are abundant in mammalian pancreatic juices and in the venoms of snakes and insects and display diverse roles, including blood platelet aggregation (Waite, 1988). They have been divided into different groups I, II, III, V, IX, X and XI based on their molecular weight, substrate specificity, amino acid sequence and disulphide bond pattern (Renetseder et al., 1985; Farooqui et al., 1992; Ackermann and Dennis 1995; Six and Dennis, 2000). Groups I, II, III, V, IX, X and XI are designated secretory PLA<sub>2</sub> (sPLA<sub>2</sub>). They are small proteins of 119 to 143 amino acids, with low molecular weights ranging between 12 and 18 kDa (Arni and Ward, 1996; Six and Dennis, 2000). These PLA<sub>2</sub>s are characterized by their dependency on millimolar calcium, and lack of preference for arachidonate over other fatty acids at the sn-2 position. They also contain disulfide bonds that are essential for catalytic activity.

Intracellular PLA<sub>2</sub>s are often membrane associated and are involved in phospholipid metabolism, signal transduction and other varied essential cellular functions (Mukherjee et

al., 1994). They have also been classified into groups IV, VI, VII and VIII according to molecular weight, substrate specificity, amino acid sequence, calcium - dependency and disulphide bond pattern. They have higher molecular weights ranging from 26 – 114 kDa requiring none or nano-molar calcium levels (Six and Dennis, 2000) for activity.

The different isoforms of PLA<sub>2</sub> have been shown to demonstrate different substrate specificities, may be localized in different cell types within the CNS, and they possess different mechanisms of activation (Farooqui et al., 1992). A comparison of the properties of the different forms of PLA<sub>2</sub> enzymes suggests that these proteins constitute a family of enzymes responsible not only for the degradation of membrane phospholipids, but also for the generation of unsaturated free fatty acids and lysophospholipids (Farooqui et al., 1997a). At physiological levels both these metabolites act as second messengers, but at high concentrations they are cytotoxic to cells acting as detergents producing changes in the activities of membrane enzymes (Weltzien, 1979; Farooqui and Horrocks, 1991). At lower concentrations, they produce changes in membrane fluidity and permeability thereby affecting normal cellular functions (Chao and Olson, 1993; Moolenaar, 1995). A second messenger is an intracellular mediator formed by the binding of an effector to a receptor. Of all the different classes the 85 kDa group IV PLA<sub>2</sub> enzyme is of particular interest because of its implications in schizophrenia.

Group IV PLA<sub>2</sub>s are intracellular enzymes that characteristically have higher molecular mass ranging from 61 - 114 kDa (Six and Dennis, 2000). These PLA<sub>2</sub> are generally localized in the cytosol, preferring arachidonate (AA) at the sn-2 position of their phospholipid moiety and resisting inactivation by reducing agents. They are designated cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>). The catalytic activity of type IV PLA<sub>2</sub>s results in the

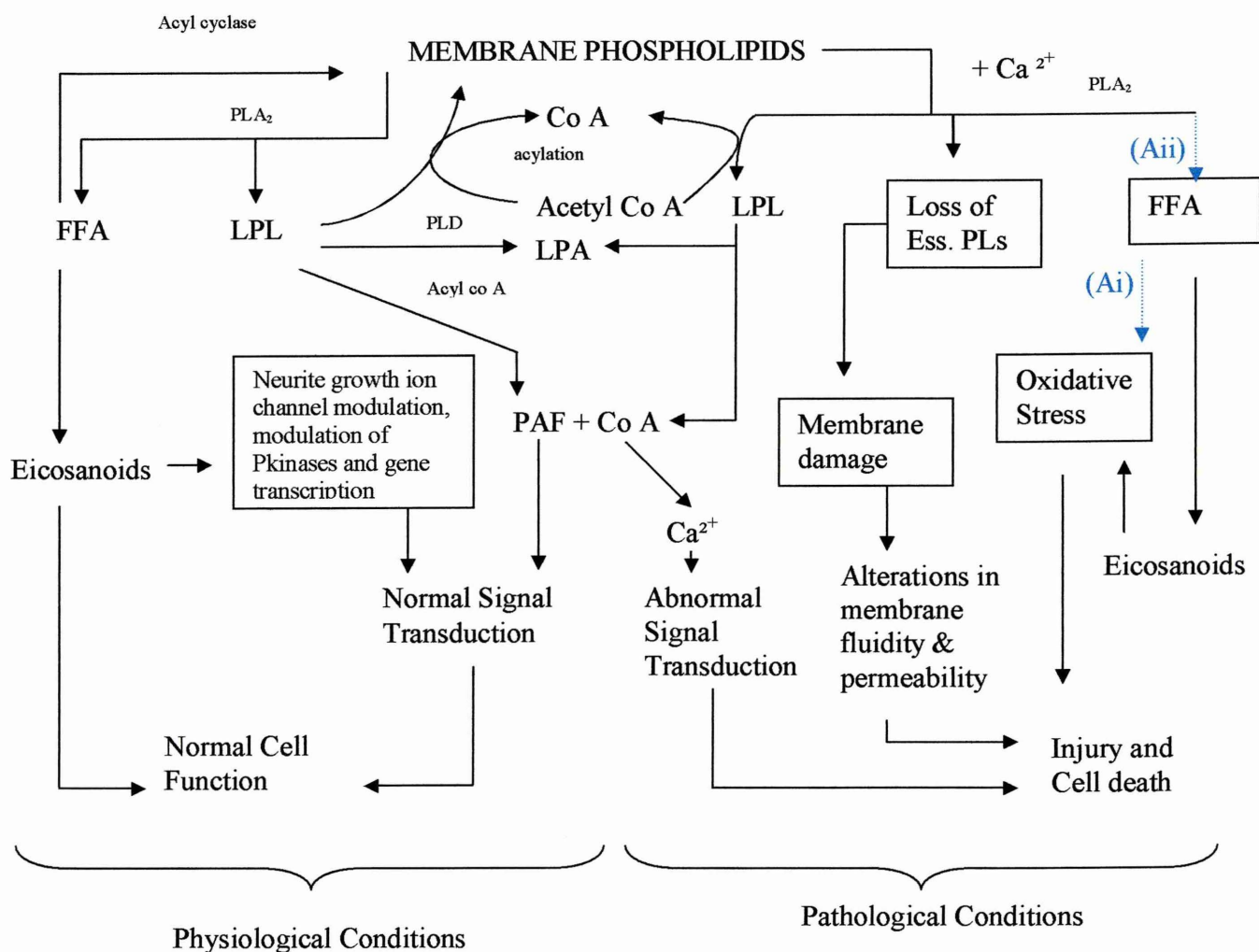
release of arachidonic acid, a precursor of eicosanoids, which is implicated in triggering inflammatory reactions (Kudo et al., 1993). Type IV PLA<sub>2</sub>s are further classified into two groups based on their calcium (Ca<sup>2+</sup>) dependency as either Ca<sup>2+</sup> - dependent enzymes requiring 10 – 1,000 nM calcium or Ca<sup>2+</sup> - independent. They have also been classified according to their substrate preference, molecular weight and pH optimum. The 85 kDa cPLA<sub>2</sub> has been purified, sequenced, and cloned from neuronal, non-neuronal tissues and the U937 human lymphoma cell line (Clark et al., 1991; Kramer et al., 1991; Stephenson et al., 1994).

Arachidonic acid has been implicated in both physiological (synaptic plasticity) and pathophysiological (neurodegenerative) processes (Katsuki and Okuda, 1995). It's functions include the modulation of ion channels and the regulation of the activity of many enzyme proteins such as protein kinase A, protein kinase C, NADPH oxidase, GTPase, and the platelet - derived growth factor receptor (Farooqui et al., 1997a). AA inhibits glutamate uptake that is mediated by excitatory amino acid transporters in intact cells, tissue slices, amino synaptosomes and various types of neuronal and glial cell cultures. (Zerangue et al., 1995).

Eicosanoids, which are derived from polyunsaturated fatty acids (AA) (Fig. 1.21), exert their pleiotropic effects on cellular functions that implicate them as important mediators of both normal homeostatic and pathological states in cellular functions. They are metabolites of AA affecting various human neutrophil functions (Goetzl and Pickett, 1981; Serhan et al., 1984 and Ford-Hutchinson, 1997). The stimulation of neutrophils with various stimuli such as zymosan (Waite et al., 1979), calcium ionophore (Stenson and Parker, 1979), and chemotactic factor (Hirata et al., 1979), triggers the release of AA from membrane

phospholipids, particularly PI and PC. PI is potentially the most important source of arachidonate, due to its unusual enrichment in this fatty acid (Holub and Kukcis 1971; Bockman et al., 1981; Majerus et al., 1984). Walsh et al., 1981, provided more evidence that the release of arachidonate from human neutrophils occurs by the action of a phospholipase A<sub>2</sub>. Normally, AA and LPLs are recycled through a series of energy - dependent reactions. As a result the normal phospholipid content of neuronal membranes is not altered and intracellular concentrations of free arachidonate and LPLs are maintained at low physiological levels.

In the human brain, cPLA<sub>2</sub> is predominantly located in the astrocytes of the gray matter (Stephenson et al., 1994). In non-neuronal tissues, cPLA<sub>2</sub> activity is regulated by phosphorylation/dephosphorylation processes involving the translocation of this enzyme from the cytosol to the plasma membrane (Kramer et al., 1993). In primary cultures of neuronal cells, arachidonic acid released from membrane phospholipids can be stimulated by several neurotransmitters including dopamine, nor-epinephrine, serotonin and glutamate (Piomelli, 1994). This release is mediated primarily by PLA<sub>2</sub> activity linked to receptors through a G-protein (Axelrod, 1990). The interaction of glutamate and N-methyl-D-aspartate (NMDA) with excitatory amino acid receptors produces a marked increase in the AA release from membrane phospholipids of the striatal, hippocampal, and hypothalamic neurons and cerebellar granule cells (Dumuis et al., 1988).



**Fig. 1.31** Functions of PLA<sub>2</sub> in the brain. (Ai) and (Aii) represent the strategic points of interest in this study for the demonstration of antioxidant and anti-PLA<sub>2</sub> activities by PUFAs and certain dietary antioxidants.

### 1.8.15 PLA<sub>2</sub> and membrane repair

PUFAs in membrane phospholipids are highly susceptible to peroxidative damage (Farooqui et al., 1994). Peroxidized PLs in cell membranes may yield a membrane-packing defect, thus making the sn-2 ester bond more vulnerable to PLA<sub>2</sub> action (Farooqui et al., 1997a). Many studies have implicated phospholipid hydroperoxides as better substrates for PLA<sub>2</sub> activity in comparison with the native (unperoxidized) phospholipids (McLean et al.,

1993). The hydrolysis of lipid peroxides removes peroxidized fatty acyl chains, which are reduced and re-esterified (van Kuijk et al., 1987). As a result, PLA<sub>2</sub> activity repairs and restores the appropriate physico-chemical state of membranes preventing the peroxidative cross-linking reactions, without which peroxidized lipids would accumulate producing devastating alterations in cell membrane functions (Farooqui et al., 1997a).

#### **1.8.16 PLA<sub>2</sub> and neurodegeneration**

The stimulation of PLA<sub>2</sub> has been reported in many neurodegenerative diseases including schizophrenia (Gattaz et al., 1995; Ross et al., 1995); ischaemia (Rordorf et al., 1991; Edgar et al., 1982) head injury (Shohami et al., 1989), spinal cord injury (Farooqui and Horrocks, 1991); and Alzheimer's disease (Stephenson et al 1996). For instance, studies have shown a significant increase in plasma and serum PLA<sub>2</sub> activity in drug-free schizophrenic patients, compared to healthy and non - schizophrenic psychiatric controls (Gattaz et al., 1987, 1990; Nojonen et al., 1993). The stimulation of PLA<sub>2</sub> coupled with the increase in the breakdown of membrane phospholipids may be harmful to neuronal cells in the following ways:

- Loss of essential membrane phospholipids resulting in the accumulation of FFA, LPL and platelet activating factors (PAFs).
- Accumulation of FFA which may set an uncontrolled "arachidonic acid cascade" in motion, thus setting the platform for increased production of free-radicals and reactive oxygen species leading to lipid peroxidation and damage to membrane proteins and even DNA.
- Calcium influx through increased phospholipid breakdown leads to sustained activation of protein kinase C (PKC) and its translocation from cytosol to plasma



membrane. The stimulation and translocation of PKC may be involved in neurodegeneration (Farooqui and Horrocks, 1991).

## **1.9 THE HUMAN BRAIN AND OXYGEN**

The brain is an extremely complex organ and the understanding of its structure and function has evolved over centuries. With the advent of tools such as neuro-imaging and neuro-physiology researchers can see the living, feeling, thinking human brain at work. The brain is responsible for merging molecular, genetic and biochemical information with information from the world.

Within the brain, there are 2 cell types: neurons (nerve cells) and glial cells. Neurons are responsible for transmitting (sending and receiving) nerve impulses or signals to and from the brain whereas the glial cells provide neurons with nourishment, protection and structural support. Collectively, there are more than 100 billion neurons in the brain, consisting thousands of distinct types. Each neuron communicates with another via specialized structures known as synapses. Over 100 distinct brain chemicals, called neurotransmitters, communicate across these synapses in the brain. The communication system or circuit, formed by hundreds or thousands of neurons is termed neurotransmission, which gives rise to complex mental behavioural responses.

During fetal development, genes drive the brain formation resulting in a specific and highly organized structure. Early developments of the brain can be influenced by environmental factors such as mother's nutrition, alcohol consumption, tobacco smoking, other psychoactive substances or exposure to radiation. After birth and throughout life, all types of life-experiences have the ability to generate immediate communication between and

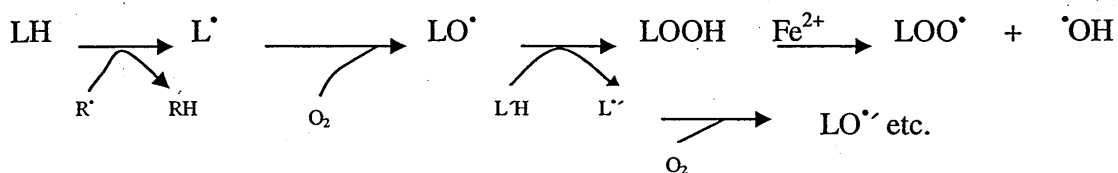
among neurons as well as to initiate molecular processes that remodel synaptic connections (Hyman, 2000). Hence, new synapses can be weakened, strengthened, created or existing ones removed. Hence, information processing within the circuit will be changed to accommodate the new experience.

The human brain requires oxygen and its mitochondrial activity is very high to meet energy demands for neuronal activity (Halliwell and Gutteridge, 1999). As a result, the brain is always under high oxidative stress. Oxidative stress is an imbalance between oxidants and antioxidants, in favour of the oxidants potentially leading to damage (Halliwell and Gutteridge, 1999). Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions. The brain is enriched with lipids particularly PUFAs, which are highly susceptible to peroxidation (Hajimohammadreza and Brammer, 1990). As much as 20% of the brain by dry weight consists of PUFAs mostly AA and DHA (Horrobin, 1996). Often electrons escape from the electron transport chain and react with molecular oxygen to generate free radicals, which can cause peroxidative damage to lipids in mitochondrial membranes. Excessive generation of free radicals may result in oxidative damage to membrane lipids, proteins or nucleic acids causing extensive cellular dysfunction or even death. Peroxidative damage to cell membranes has been implicated in the neuropathophysiology of many psychiatric illnesses including schizophrenia and Alzheimer's disease (Reddy and Yao, 1996; Peet, et al., 1999).

## **1.10 LIPID PEROXIDATION**

The brain is particularly vulnerable to free radical damage as it is a highly oxygenated organ utilizing 20% of the total oxygen required by the body (Lambertsen, 1980). In

addition, it is enriched with iron and PUFAs (O'Brien and Sampson, 1965) and relatively poor in antioxidant enzymes such as catalase (Halliwell and Gutteridge, 1986; Halliwell 1989). PUFAs are highly susceptible to attack by free radicals (Bielski et al., 1983) due to lowered bond dissociation energy of their allylic hydrogens (Fig. 1.32).



**Fig. 1.32** The lipid peroxidative process. LH: lipid; L•: lipid radical; LO•: lipid peroxyl radical; LOOH: lipid hydroperoxide; LOO•: lipid hydroperoxyl radical; •OH: hydroxyl radical; R•: substrate radical and RH: reduced substrate.

The degenerative propagation reactions in lipid membranes are usually accompanied by the formation of a wide variety of products, including alkanes, aldehydes (such as hydroxy alkenals) and carbonyl compounds. Hydroxy alkenals are toxic by themselves; they may serve as second messengers for radical damage (Humad et al., 1988). Peroxidative damage to neuronal cells may affect membrane transport systems and permeability causing loss of mitochondrial energy production, gene expression and therefore receptor-mediated phospholipid – dependent signal transduction, which may explain the altered information processing observed in schizophrenia (Mahadik et al., 2001).

### 1.10.1 Free radicals

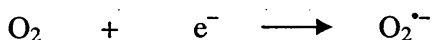
Free radicals, some of which are also referred to as reactive oxygen species (ROS), play an essential role both in human health and disease. They are generated under normal physiologic conditions during aerobic metabolism (Mahadik and Mukherjee, 1996a). They are extremely important in normal metabolic processes. A small proportion of these

radicals have physiologic roles but the remaining are inactivated by antioxidant enzyme systems (Burton and Ingold, 1989; Halliwell and Gutteridge, 1990a). Free radicals are generally very reactive molecules possessing an unpaired electron. The life of a free radical has three stages: initiation, propagation and termination.

Free radicals have 2 major sources: endogenous and exogenous. Endogenous sources of free radicals include those generated intracellularly, acting within the cells, and those formed within the cell but released into the surrounding area. Exogenous sources include irradiation, alcohol, solvents and some medication such as anti-neoplastic drugs, some antibiotics and anesthetics (Halliwell and Gutteridge, 1986). Free radicals may be produced by the activation of phagocytes and the immune system as a whole, lipid peroxidation, electron transfer system in the mitochondria, ischaemia, and trauma (McCord, 1987; Gutteridge, 1995). These intracellular free radicals may occur as a result of the activity of certain oxidases, lipoxygenases, cyclooxygenases, dehydrogenases and peroxidases. Electron transfer from metals such as iron to oxygen - containing molecules can also initiate free radical reactions. A wide range of free radical molecular species is endogenously formed. The singlet oxygen is not a free radical but nevertheless a reactive oxygen species capable of causing cellular damage (Machlin and Bendich, 1987).

Not all free radicals cause fatal injury. For example, white blood cells involved in the immune response of the body produce and mobilize oxygen-containing free radicals to destroy fungi, viruses and other foreign bodies they ingest. Furthermore, free radicals govern the production of complements and prostaglandins, which are vital to the protection and physiological functions of the body. Also, the liver utilizes free radicals in the process of detoxification. Free radicals may be generated by normal catalytic reactions by the

activities of enzymes and transport molecules, for example, xanthine oxidase and aldehyde oxidase. These two enzymes generate the superoxide anion radical by the addition of a single electron to molecular oxygen (Fig. 1.33).



**Fig. 1.33** The generation of the superoxide anion radical (mitochondrial error).

Cellular mitochondria are the major sources of endogenous free radical utilized in the synthesis of ATP from ADP, the primary energy currency of the body. Auto-oxidation reactions produce free radicals from the spontaneous oxidation of biological molecules involved in non-enzymatic electron transfers. Examples of compounds that may undergo auto-oxidation in the body include: thiols, hemoglobin and catecholamines. In all of these auto-oxidation reactions, superoxide is the main free radical species produced initially.

Metals, such as copper, iron, mercury, beryllium and silver, may produce free radicals and are believed to derive their toxic effects from their inherent ability to transfer electrons, thus generating free radicals (Miller et al., 1990). These free radicals can adversely affect cellular health by producing lipid peroxidation of intracellular membranes and cross linkages of membrane macromolecules (Potwarka, 1999). When irradiated water is ionized, an electron is removed from the molecule, leaving an ionized water molecule. The species resulting from the radiolysis of water are the free radicals  $\text{H}^\bullet$  and  $\text{OH}^\bullet$  and  $\text{e}^-_{(\text{aq.})}$  (hydrated electrons). They are highly reactive having a lifetime of  $10^{-9} - 10^{-11}$  seconds. The hydroxyl radical ( $\text{OH}^\bullet$ ) is extremely reactive. Water presents the largest number of target molecules in a cell; most of the energy transfer goes on in water when a cell is irradiated. Oxygen is an excellent electron acceptor and can combine with  $\text{H}^\bullet$  to form a

peroxyl radical ( $\text{HO}_2^\bullet$ ). Free radicals have a relatively short half-life, therefore the determination of their levels can be difficult.

### **1.10.2 Free radical damage**

Free radical damage may involve any cell. The human body contains an estimated 100 trillion cells, each containing various organelles, all having specific functions. The organelles are membrane-bound complex bodies. The membranes surrounding these organelles are vital dynamic structures important in controlling the passage of nutrients and excretory products involved in cellular metabolism. They are also necessary for the organization of cellular enzymes. When highly reactive free radicals come in contact with these membranes, they can initiate lipid peroxidation and membrane destruction (Mahadik et al., 1994), which may result in the loss of the organization of cellular enzymes, a disturbance in the distribution of nutrients and even cellular metal dysfunction, leading to degenerative disease processes. The health problems associated with alcohol consumption and smoking, are related to free radical damage (Davidson et al., 1998; Zimmer et al., 1998; Yao et al., 2000). Copper, manganese, iron and other trace redox - active transition metals have been implicated in the neuropathology of schizophrenia (Gattaz et al., 1990). These metals are essential in most biological reactions (e.g. in the synthesis of DNA, RNA and proteins) and as co-factors of various enzymes, particularly those involved in respiration, thus their deficiency can lead to disturbances in the central nervous system and other organ functions. Excessive tissue accumulation of redox-active transition metals can be cytotoxic particularly because perturbances in metal homeostasis result in an array of cellular disturbances characterized by oxidative stress and increased free radicals production.

The total number of free radicals is unknown. An estimated 10,000 free radicals bombard each body cell daily. This amounts to a high free radical activity since the body contains about 100 trillion cells. As such, if all free radicals were deadly, humans would cease to exist. Antioxidants, present as cellular components, delay or even inhibit the damaging effects of free radicals (Halliwell and Gutteridge, 1999). An antioxidant is any substance that, when present at low concentrations compared to that of the oxidizable substrate, significantly delays or prevents the oxidation of that substrate (Halliwell and Gutteridge, 1999).

### **1.11 BIOMARKERS OF LIPID PEROXIDATION**

Oxidative stress induced by the action of free radicals or reactive oxygen species (ROS) in biological membranes produce several by-products (also known as biomarkers). Lipid peroxides are the major products resulting from free radical damage. In humans and animals, lipid peroxides are degraded into a wide variety of products such as:

- Volatile hydrocarbons (alkanes) measurable in exhaled air. For example, ethane and pentane. The measurement of alkanes as an index of lipid peroxidation was first suggested by Riely et al. (1974). They investigated the relationship between tetrachloromethane administration and ethane exhalation in mice. Kivits et al. (1981) demonstrated that n-3 PUFA oxidation resulted in increased ethane excretion, while n-6 PUFA oxidation yielded increased excretion of pentane. n-3 and n-6 are the most abundant PUFAs in cell membranes.
- Aldehydic products, such as hydroxy alkenals and malonyldialdehyde (MDA). These are also known as Thiobarbituric acid reactive substances (TBARS) (Humad, 1988). In vitro studies using microsomes incubated with  $\text{Fe}^{2+}$ /ADP showed the formation of MDA (Frank et al., 1980).

- Isoprostanes. These are a group of prostaglandin (PG) - like compounds recently discovered. They are produced by a reaction between AA and free radicals (Morrow et al., 1990).

As the name implies, antioxidants combat oxidative stress. They prevent rancidity. They protect against damage by free radicals. When free radicals are generated in the living system, a wide variety of antioxidants come into play: enzymatic and non-enzymatic antioxidants.

The composition of antioxidant defenses differs from cell to cell; tissue to tissue and between organisms. Antioxidant activity may be accomplished by inhibiting the initiation of the free radical; directly or indirectly scavenging generated free radicals; or by raising the endogenous antioxidant defenses (that is by increasing expression of genes coding the antioxidant enzymes).

## **1.12 ANTIOXIDANTS**

There are two major classes of antioxidants: the enzymatic and non-enzymatic.

### **1.12.1 Enzymatic antioxidants**

All eukaryotic cells contain powerful antioxidant enzymes. The three major classes are: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx).

SOD is widely distributed in the human brain and in virtually all eukaryotic cells (Halliwell and Gutteridge, 1999). Its activity steadily increases with age. There are three types of SOD. One is the copper-zinc metallo-enzyme, predominantly located in the cytosol, but



some appear to be present in lysosomes, peroxisomes, nucleus and the inter-membrane space of the mitochondria (Fridovich, 1995). Another is the manganese metallo-enzyme predominantly localised in the mitochondria (Mahadik and Mukherjee, 1996b). The third is the iron containing metallo-enzyme, which has never been found in animal tissues. It is present in some bacteria and some higher plant tissues (Halliwell and Gutteridge, 1999). SOD converts the superoxide anion radical ( $O_2^{\cdot-}$ ), in the presence of substrates providing protons, to hydrogen peroxide ( $H_2O_2$ ) (Mahadik and Scheffer, 1996). The ubiquitous location of SOD provides sufficient protection against the superoxide anion radical during normal physiological conditions.

CAT is primarily localized in peroxisomes, lysosomes, and mitochondria (Vitorica et al., 1984). It is a heme-containing metallo-enzyme, which converts hydrogen peroxide formed by SOD to water and molecular oxygen. Hydrogen peroxide ( $H_2O_2$ ) is toxic and when present in sufficient quantities can interfere with normal metabolic reactions. CAT is considered to be ineffective for protection against free radical damage to cytosolic and nuclear components, where  $H_2O_2$  is spontaneously converted into hydroxyl radicals ( $\cdot OH$ ) in the presence of transition metals (Mahadik and Scheffer, 1996). It is believed that low levels of CAT contribute to oxidative stress by  $H_2O_2$  in peroxisomal disorders (Mahadik and Scheffer, 1996).

GSHPx is predominantly localized in the cytosol with low levels in the mitochondria. It has high levels in neuronal cell areas prone to oxidative damage (Savolainen, 1978; Brannan et al., 1980). GSHPx is a selenium containing metallo-enzyme (Cohen and Hochstein, 1963). It constitutes a very small proportion of brain selenium and its levels are influenced by dietary availability of selenium (Rafferty et al., 2003). GSHPx effectively

protects against cytosolic damage by eliminating  $\text{H}_2\text{O}_2$  and lipid peroxides by reduction using glutathione.

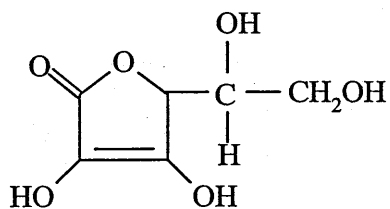
Brain levels of both CAT and GSHPx are low, while SOD levels are high. Since, the brain contains oxy-radical sensitive polyunsaturated fatty acids (Cohen and Greenwald, 1983; Martin et al., 2002), the brain is particularly exposed to oxidative injury (Ames et al., 1981).

### **1.12.2 Non-enzymatic antioxidants**

These exist in the form of nutrients taken up only from the diet. Examples of these are: vitamin C, vitamin E, and the carotenoids.

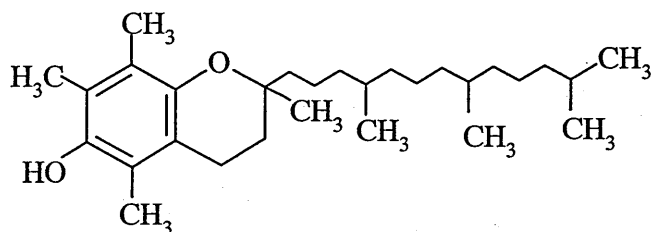
Vitamin C is also known as ascorbate because it exists in the ionized form under physiological conditions. It is a water-soluble antioxidant functioning more in the aqueous environment. Sources in the diet include capsicums, vegetables and citrus fruits. Ascorbic acid scavenges the  $\text{O}_2^{\cdot-}$ ,  $\text{HO}_2^{\cdot}$  and  $\text{OH}^{\cdot}$  radicals. It inhibits lipid peroxidation by hemoglobin - or myoglobin -  $\text{H}_2\text{O}_2$  mixtures and is a powerful quencher of singlet oxygen. Vitamin C inhibits oxidative damage by scavenging radicals generated from certain drugs such as phenylbutazone (Halliwell and Gutteridge, 1999).

Vitamin C (Fig. 1.34) has a dichotomous action in the body (Halliwell and Gutteridge, 1999). In excessive amounts, it may act as a pro-oxidant in the presence of transition metals such as copper and iron.



**Fig. 1.34** Ascorbic acid (vitamin C).

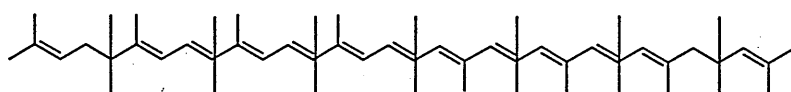
The principal role of vitamin E ( $\alpha$  - tocopherol) is to protect tissues against destructive oxidation (Burton et al., 1983; Pryor, 1984). Sources in the human diet include leafy vegetables, nuts and oils. Vitamin E inhibits lipid peroxidation by scavenging peroxy radicals. It is fat-soluble and functions mostly in the hydrophobic environment of the human cell (Burton et al., 1983).



**Fig. 1.35** Vitamin E ( $\alpha$  - tocopherol).

Carotenoids are plant-derived pigments found in animals and microorganisms. They occur widely in bacteria, fungi and algae, where they can be useful taxonomic markers. Carotenoids occur extensively in marine invertebrates. They give bright colours to any substance in which they occur. Sources of carotenoids include tomatoes, carrots, broccoli and sea foods.

The carotenoids are isoprenoid compounds biosynthesised by tail-to-tail linkage of two C<sub>20</sub> geranyl-geranyl diphosphate molecules. This produces the parent C<sub>40</sub> carbon skeleton from which all the individual variations are derived (Fig.1 36).



**Fig. 1.36** The 40-carbon skeleton from which all carotenoids are derived. The broken lines represent the C<sub>5</sub> (isoprene) units.

They are a group of hydrophobic molecules with little or no solubility in water. They are thus expected to be restricted to hydrophobic areas in the cell, such as the inner core of membranes, except when associated with proteins which allow them access to an aqueous environment (Britton, 1995). *In vivo*, the overall shape, size and hydrophobicity of a carotenoid are obviously major features that determine the ability of a carotenoid to fit into sub cellular structures. Polar functional groups provide a focus for interactions with more polar molecules in order to allow the carotenoid to participate in events in an aqueous subcellular medium or at an interface or membrane. Being highly hydrophobic, carotenoids show a strong tendency to aggregate and crystallize in aqueous media. The accumulation of carotenoids as micro-crystalline aggregations is common in the chloroplasts of higher plants (e.g. lycopene in tomato) (Pfander et al., 1994). Aggregation changes the physical properties of carotenoids (light absorption and chemical reactivity). Changes in carotenoid size and ease of solubilization affect the ease of absorption and bio-availability in animals and their ability to enter and function in sub-cellular structures (Gruszecki and Sielewiesiuk, 1990).

About 600 carotenoids have been isolated from natural sources (Stahl, and Sies, 1996) all classified under two groups of carotenoids namely:

- Hydrocarbons called carotenes such as  $\alpha$  - and  $\beta$  - carotene.  $\beta$ -carotene is converted to vitamin A (Moore, 1957) in the liver and the intestine (Krinsky et al., 1993). About 50 carotenes are known to have pro-vitamin A activity (Olson, 1989).
- Oxygenated carotenoids called xanthophylls such as lutein, cryptoxanthin, canthaxanthin (CX), zeaxanthin (ZX) and asthaxanthin (AX).

A large proportion of the known carotenoids have antioxidant activity and may also be involved in enhancing immune responses, gap junction and carcinogen metabolizing enzyme activity (Wang, 1994; Stahl et al., 1997). Carotenoids have been reported to have the ability to quench singlet oxygen and peroxy radicals (Krinsky, 1989; Edge et al., 1997).

Since oxidative stress places a burden on the entire cell / organ, it is not surprising that stress in one cell affects the redox balance in other cells. This same principle applies to antioxidants.

### **1.12.3 Combination of antioxidants**

It has been reported that dietary  $\beta$ -carotene can mediate an increase in the activities of the antioxidant enzymes: SOD, and CAT induced by high fat diets (Blakely et al., 1988). The concept that antioxidants interlock in their protective effects and that the levels of all the antioxidants in the network are important, rather than just a single antioxidant, raises the possible importance of supplementing with an antioxidant cocktail rather than a single antioxidant (Keaney and Frei, 1994).

Polar carotenoids such as AX are more effective in situations where free radical attack occurs at the liquid/aqueous interphase. However, if radicals were generated in the lipid phase, less polar carotenoids such as  $\beta$ -carotene will yield more antioxidant effect (Ojima et al., 1993). However, it has been reported that when different antioxidants are present together, there is an additive effect for conferring resistance to lipid peroxidation (Packer, 1993). This effect could be due to the fact that the different antioxidants protect at different physical locations within the cell membranes rather than physical interactions between antioxidants. Vitamin E for example, mitigates oxidation at the outer surface while carotenoids and vitamin A are protective in the interior of the membrane (Niki et al., 1995a).

Furthermore,  $\alpha$ -tocopherol prevents auto-oxidation of carotenoids at physiological concentrations (Handelman et al., 1991) and can scavenge the carotenoid peroxyl radical before it propagates peroxidation (Niki et al., 1995a). Conversely, protection of vitamin E by carotenoids may also be important when singlet oxygen attack is a factor (Ojima et al., 1993).

### 1.13 AIMS

**The main aim** was to investigate the influence on PLA<sub>2</sub> activity and antioxidant efficacy exerted by a range of essential polyunsaturated fatty acids and antioxidants, which will allow advancement in the progress towards **the ultimate aim** of increasing the understanding of the mechanism of action of EPA and other PUFAs in the treatment of schizophrenia and possibly other related neuropsychiatric disorders.

The human histiocytic lymphoma cell line (U937) was chosen as a model for the research for the following reasons: (i) previous studies on schizophrenic skin macrophages suggested reduced availability of arachidonic acid owing to an increase in PLA<sub>2</sub> activity (Ward et al., 1998), (ii) head space analysis has previously been used to detect volatile product of lipid peroxidation, in U937 cells (North et al., 1994), (iii) PLA<sub>2</sub> has been purified and characterized in U937 monocytes (Kramer et al., 1991) and (iv) U937 cells have very low levels of polyunsaturated fatty acids (Obermeier et al., 1995) and high PLA<sub>2</sub> expression (Kramer et al., 1991), thereby, mimicking the conditions reported in schizophrenic patients.

The Highland Psychiatric Research Foundation (HPRF), Inverness, was active in studying the clinical use of antioxidants and phospholipase A<sub>2</sub> inhibitors in the treatment of psychiatric illnesses. The biotechnology group of the Scottish Association for Marine Science (SAMS), Oban, was interested in looking for new products of marine organisms containing a wide range of carotenoids. Also, collaborative research was performed with The Victoria Infirmary, Glasgow Southern NHS Trust, Glasgow.

#### **1.14 OBJECTIVES**

This study (i) measured oxidative stress after challenging U937 cells with oxidants (ii) determined if oxidative stress increases fatty acid uptake into phospholipids or phospholipid breakdown into fatty acids (iii) determined the effects of PUFAs especially EPA and DHA on the uptake and release of AA (iv) determined PLA<sub>2</sub> activity/concentration and oxidative stress response of U937 cells to pre-treatment with PUFAs and several antioxidants (v) compared cell response after pre-treatment with PUFAs with/without antioxidants (vi) determined the effects of oxidative stress on PLA<sub>2</sub>

expression and (vii) established the existence of coupling *in vitro* between PLA<sub>2</sub> activation and oxidative stress.

### **1.15 HYPOTHESES**

- (1) Oxidative stress increases fatty acid release and reduces the uptake of cellular phospholipids.
- (2) PLA<sub>2</sub> activity and expression is enhanced with oxidative stress.
- (3) Pre-treatment of U937 cells with PUFAs, especially EPA, increases the uptake of AA but decreases its release.
- (4) EPA but not DHA inhibits oxidative stress in U937 cells.
- (5) Dietary antioxidants inhibit oxidative stress and reduce AA release.
- (6) Combinative treatments of U937 cells with the dietary antioxidants especially the carotenoids inhibit the over-expression and activity of PLA<sub>2</sub>.



## CHAPTER 2: U937 CELL COUNTS AND VIABILITY MEASUREMENTS

### 2.1 INTRODUCTION

Cells *in vivo* grow in a highly controlled and complex environment. On removal from their natural environment, they are dispersed from a histological configuration, where cell contact plays an important role, into a simplified growth medium. Cell culture lacks neuronal and hormonal regulation and the other intermediate metabolites present in fluids *in situ*. Cells in culture are in a foreign environment, therefore, stress conditions can only be minimized by attention to environmental optimization, an important factor being the nutritional condition, replacing the natural environment. This environment affects all physiological and metabolic events. Many nutrients have been identified as being essential for cell growth, for example, glutamine and glucose have been associated with playing key roles in cell behaviour. In addition to nutrient balance many physicochemical factors such as agitation, mixing and oxygen supply are of crucial importance to *in vitro* cell health and viability.

In this study, the U937 monocytic cell line is used as a model for neuronal lipid metabolism and breakdown. U937 is a well-characterized human lymphoma histiocytic cell line of Caucasian origin. Monocytes are cells with a diameter of 14 to 20 microns (van Furth et al., 1970) and a large folded nucleus. Mammalian cells can generally be preserved for extended periods by storage in liquid nitrogen (LN<sub>2</sub>), recovered and re-established in culture. For optimal recovery of viable cells, rapid thawing and immediate dilution in culture medium is required to reduce the toxic effects of the cryopreservative, dimethyl sulphoxide (DMSO). When cells are established in culture, they divide and the density increases. For optimal growth, the cells should not be overcrowded. The culture medium must be renewed or replaced depending on the optimal cell density for the cell type in use.

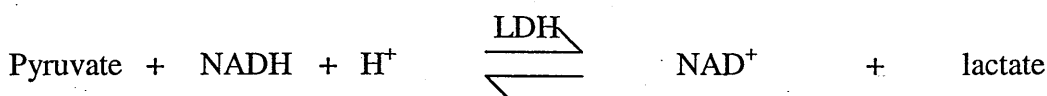
To measure the performance, achieve reproducibility or make comparative studies, a method of quantifying cell population is required. The method used here utilizes a microscopic counting chamber (haemocytometer) and is simple, quick, cheap and requires only a small fraction of cells from a suspension. There are several types of haemocytometers on the market. The Improved Neubauer has proved to be the most popular. A thick, flat cover slip rests on the counting chamber at a distance of 0.1 mm above the slide base, with two chambers. The slide base has accurate engraved rulings on it, comprising 1 mm squares, some of which are further divided into smaller squares. This method is based on the principle that live cells can be differentiated from dead cells using the physical property of refractive index.

To ascertain that a cell culture has reached its optimum level of growth before routine sub-culturing and storage, before and after the addition of certain potentially toxic chemicals, it is necessary to obtain a measure of the viability and cell population. The screening of agents that may be cytotoxic to cells is of great importance prior to experiments. Testing the toxicity of water-soluble compounds presents no inherent problems regarding extraneous additives but testing of water-insoluble agents generally require the inclusion of various often toxic solvents. Thus it is important to know the maximum tolerable concentration of the potentially toxic bio-active compounds that will be used in this study on the *in vitro* system employed. Two different methods of cell viability measurements are used in this study, both well established. One is the Trypan Blue dye exclusion method and the other is the lactate dehydrogenase (LDH) activity method.

Trypan Blue is a staining dye distinguishing viable from non-viable cells. Trypan Blue acts by penetrating cells with lost membrane integrity. As such non-viable cells are

stained blue, granular and /or fragmented while viable cells remain unstained, intact and refractive. The use of stains such as Trypan blue are subjective and cannot give absolute values, cell numbers and take no account of differences in cell size/mass. This method is quick and cheap requiring only a small fraction of the total cells from a cell suspension.

The LDH activity method measures lactate dehydrogenase, an intracellular enzyme present in culture supernatants due to leakage, giving a quantitative value for the loss of cell viability. It is a non-invasive method of cell viability measurement. The principle is based on the following reaction:



The activity of LDH can be measured as the reduction of pyruvate to lactate (Vassault, 1983). The reduction is coupled to the oxidation of NADH to NAD<sup>+</sup>, which is measured spectrophotometrically at 340 nm. The equilibrium is on the side of NAD<sup>+</sup> and lactate. Due to the fact that NADH has a high absorbance at 340 nm compared to NAD<sup>+</sup>, the reaction is measured as the rate of decrease in absorbance at 340 nm. This study utilizes a colorimetric LDH assay kit (Sigma) based on the reduction of a tetrazolium dye. The LDH kit used measures cell membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. In other words, it measures LDH leakage into the cell medium. The resulting coloured compound is measured spectrophotometrically at 490 nm.

This study utilizes the Trypan Blue method consistently for cell counts and viability determinations prior to cell treatments with the various agents. After ensuring consistent and reproducible results between Trypan Blue and LDH viability methods,

the LDH activity assay was chosen over the Trypan Blue method because of its non-invasive nature and speed of analysis.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Materials**

Hanks balanced salt solution (HBSS), RPMI 1640, heat-inactivated foetal bovine serum, gentamicin solution (50 mg/ml), dimethyl sulphoxide (DMSO), new-born calf serum (NCS), 30% (w/w) hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), fatty-acid-free bovine serum albumin (BSA), iron sulfate ( $\text{FeSO}_4$ ), 70% tert-butyl hydroperoxide (t-BHP), HPLC grade methanol (MeOH), ethanol (EtOH), chloroform ( $\text{CHCl}_3$ ) and hexane ( $\text{C}_6\text{H}_{14}$ ), stearic acid (SA), oleic acid (OA), arachidonic acid (AA), ascorbic acid, vitamin E,  $\beta$ -carotene, astaxanthin, 0.4% Trypan Blue solution and LDH (TOX-7) assay kit were purchased from Sigma-Aldrich, UK. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were obtained from Nu-chek Prep., Denmark. U937 cells were a kind gift from the Scottish Antibody Production Unit (SAPU), Lanarkshire, Glasgow.

### **2.2.2 Glassware and plastic preparations**

All glassware used was washed in De-con and nano-pure water, autoclaved at  $121^\circ\text{C}$  for 30 min and dried before use. All other apparatus were sterile before use with all experiments performed under a laminar flow hood (class II).

### **2.2.3 U937 cell culture**

#### **2.2.3.1 Thawing of cells**

U937 cells used in this study were gifted as 1 ml frozen samples from SAPU, Glasgow. A 1 ml vial containing  $1.0 \times 10^6$  cells/ml was thawed rapidly in a water bath set to  $37^\circ\text{C}$ . The vial was observed and removed from the water bath as soon as two-thirds of the

medium thawed. The vial was disinfected by spraying with 70% ethanol (EtOH) and wiped dry with a paper towel. Its contents were transferred into a 15 ml centrifuge tube containing 10 ml of ice-cold Hanks balanced salt solution (HBSS) in a class II laminar flow hood. The tube contents were mixed thoroughly by careful inversion and centrifuged at 250 g for 5 min at 4°C. Cell supernatant was discarded into a sterile beaker before the addition of 10 ml of freshly prepared culture medium (CM). Culture medium contained RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 0.05 mg/ml gentamicin. Using a sterile plugged glass Pasteur pipette, suspended cells were transferred into a 25 cm<sup>2</sup> culture flask and placed in a CO<sub>2</sub> incubator set to 5% CO<sub>2</sub>/95% air and 37°C in a humid environment for 24 h. The flask cap was loosened sufficiently to allow gas exchange.

#### ***2.2.3.2 Continuous culture of cells***

Cells, in suspension, were inspected microscopically and discarded if there was any sign of bacterial contamination. Bacterial contamination was initially detected by a colour change in the cell medium and under the microscope for movements. Otherwise, cells were transferred from the 25 cm<sup>2</sup> culture flask into a 75 cm<sup>2</sup> flask containing 10 ml of freshly prepared CM. Cells were counted every 2 days and maintained at viability > 85% and density between 0.5 and 1.0 x 10<sup>6</sup> cells/ml by the addition or replacement of fresh CM. Addition of culture medium was achieved by splitting flask contents depending on the cell density and supplementing with fresh medium. The replacement of culture medium was by centrifuging cell suspensions, discarding supernatant and re-suspending cells in fresh CM. Microscopic examination of cells was performed daily.

#### ***2.2.3.3 Storage of Cells***

0.2 – 0.4 x 10<sup>6</sup> cells/ml with viability > 90% were transferred into appropriate sterile centrifuge tubes and centrifuged at 250 g for 5 min at 4°C. Supernatants were discarded and without any delay, cells were re-suspended in 1 ml of ice-cold freezing medium (FM), which contained 90% newborn calf serum (NCS) and 10% dimethyl sulphoxide (DMSO). Cells were transferred rapidly into labelled 1 ml sterile vials and immediately frozen at –80°C. After 24 – 48 h, vials were transferred into –200°C liquid nitrogen (LN<sub>2</sub>) freezers until needed. Cells from one vial were re-established in culture to ensure the viability of the working cell bank (WCB).

#### ***2.2.3.4 Cell counting***

A homogenous suspension of cells to be counted was prepared by careful aspiration and dispensing using a Pasteur pipette, noting the volume. The haemocytometer and cover slip were cleaned by spraying with 70% EtOH and dried with a paper towel. The edge of the cover slip was moistened and placed centrally over the counting area and across the grooves. Gently and lightly, the cover slip was moved back and forth over the chamber until Newton rings appeared, indicating that the cover slip was in the correct position to allow accurate counting i.e. the depth of the chamber was now 0.1 mm. After ensuring a homogenous cell mixture, a small amount of cell suspension was transferred to both chambers of the haemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action. Using a light microscope at low power, focussed on the chamber, all the cells in each of the 25 center squares were counted using a tally counter, one side of the chamber at a time. This procedure was repeated to get 4 readings. The total cell count is equal to average count x volume of cell suspension x 10<sup>4</sup> (where 10<sup>4</sup> is the conversion factor from 0.1 mm<sup>3</sup> to ml).

**2.2.4 Cell viability measurements**

**2.2.4.1 Trypan Blue dye-exclusion method**

An aliquot of cell suspension was taken after careful mixing into 0.5 ml eppendorf tubes containing equal volumes of 0.4% Trypan Blue solution and mixed by aspiration and dispensation using a Pasteur pipette. A sample was drawn and transferred to each of the haemocytometer counting chambers. Using an inverted light microscope at low power, the number of stained and un-stained cells was counted.

**Table 2.1** Trypan Blue dilutions.

Cells	Trypan blue	Dilution factor
0.1 ml	0.1 ml	2 - fold
0.1 ml	0.3 ml	4 - fold

Total number of viable cells =  $A \times B \times C \times 10^4$ .

Total number of non-viable cells =  $A \times B \times D \times 10^4$ .

Total number of cells =  $A \times B \times (C + D) \times 10^4$ .

Where A represents the volume of cell suspension; B, the dilution factor of Trypan blue (Table 2.1); C, the average number of unstained cells; D, the average number of stained cells and  $10^4$ , the conversion factor from 0.1 mm<sup>3</sup> to 1 ml.

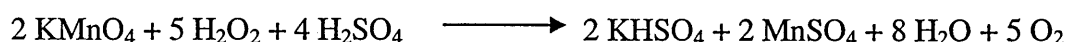
Cell viability was expressed as the ratio of the number of unstained cells to the sum of stained and unstained cells. Throughout this study, an aliquot of cell suspension was diluted in a 1: 1 (v/v) ratio with 0.4% Trypan Blue and counted with a haemocytometer.

#### **2.2.4.2 Lactate dehydrogenase (LDH) activity assay**

As instructed on the Sigma LDH assay kit (TOX-7) product, cultures were removed from the incubator into the laminar flow hood and centrifuged at 250 g for 5 min at 4°C. Background absorbance of an empty, sterile 96-well plate was measured at 690 nm using the EL 340 microplate reader. A 50 µl aliquot of cell supernatant was transferred into each well of the plate before the addition of 25 µl of the LDH assay mixture using a multi-channel pipette. The LDH assay mixture contains equal amounts of the LDH assay substrate, enzyme preparation and dye solution and was prepared just before use. The plate was covered with an aluminium foil and placed in a dark cupboard for 25 min. The reaction was terminated by adding 7.5 µl of 1 N HCl to each well using a multi-channel pipette and the absorbance was immediately measured spectrophotometrically at 490 nm. The final absorbance readings were expressed as the difference between the absorbance readings at 490 nm and 690 nm ( $Ab_{490\text{ nm}} - 690\text{ nm}$ ).

#### **2.2.5 Analysis of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)**

Initial concentrations of hydrogen peroxide were analysed by the method of Treadwell and Hall (1924). 1 ml of 30% (w/w) H<sub>2</sub>O<sub>2</sub> was added to 99 ml of water in a 100 ml volumetric flask and mixed thoroughly. 10 ml of the resulting solution was transferred into a clean beaker and diluted to a final volume of 350 ml before the addition of 25 ml of 7.5 N sulphuric acid (H<sub>2</sub>SO<sub>4</sub>). The mixture was titrated with freshly prepared 0.1 N potassium permanganate (KMnO<sub>4</sub>) until the formation of a permanent pink colour. This method was based on the following reaction:





Molar calculations from the above equation:

$n_2M_1V_1 = n_1M_2V_2$  (where “n” represents the molar ratios, “M”, the molar concentrations, “V”, volume (ml) and subscripts 1 and 2 representing  $\text{KMnO}_4$  and  $\text{H}_2\text{O}_2$ , respectively). Therefore,  $M_2 = (n_2M_1V_1)/(n_1V_2)$ .

## 2.2.6 Cell treatments

### 2.2.6.1 With Oxidants

#### (A) With Hydrogen peroxide ( $\text{H}_2\text{O}_2$ )

The concentration of  $\text{H}_2\text{O}_2$  was determined by titration with  $\text{KMnO}_4$  as earlier described (2.2.5). A stock solution of  $\text{H}_2\text{O}_2$  was prepared at a concentration of 10 mM. To obtain 0 to 1000  $\mu\text{M}$  final concentration of  $\text{H}_2\text{O}_2$  in cell suspensions, 0 to 100  $\mu\text{l}$  of the stock solution was further diluted with CM to a final volume of 0.9 ml. U937 cells were washed twice in HBSS (by centrifugation) and re-suspended in CM. 0.1 ml aliquots of cell suspensions, each containing  $0.5 \times 10^6$  cells/ml and viability > 85% were transferred into each well of a 24-well plate and incubated with 0.9 ml of  $\text{H}_2\text{O}_2$  solution in CM at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in a humid environment.  $\text{H}_2\text{O}_2$  concentration ranged from 0 – 1 mM. All cells used were from the same population. After incubation for 24 h, cells were transferred into 1.5 ml eppendorf tubes and 10  $\mu\text{l}$  aliquots taken for Trypan Blue analysis (2.2.4.1). Thereafter, the cells were centrifuged at 250 g for 5 min at  $4^\circ\text{C}$  and aliquots of cell supernatant taken for LDH analysis (2.2.4.2).

#### (B) With tert- butyl hydroperoxide (t-BHP) : iron sulfate ( $\text{FeSO}_4$ )

Cells were counted as previously described (2.2.3.4), adjusted to contain  $0.5 \times 10^6$  cells/ml, washed twice in HBSS (by centrifugation) and re-suspended in HBSS. 0.1 ml aliquots of cell suspensions each containing  $0.5 \times 10^6$  cells were transferred into each well of a 24-well plate. Treatments were started by the addition of 0.9 ml of freshly

prepared oxidant solution in HBSS or HBSS only as the control. The oxidant contained a mixture of tert butyl hydroperoxide (t-BHP) and iron sulfate ( $\text{FeSO}_4$ ) in a 1: 1 molar ratio prepared from stock solutions of 2 mM t-BHP and 5 mM  $\text{FeSO}_4$  in HBSS. Cells were then incubated at 37°C and 5%  $\text{CO}_2$  in a humid environment. For the dose-dependent experiment, final t-BHP :  $\text{FeSO}_4$  molar concentrations were varied from 50 : 50 to 1000 : 1000 ( $\mu\text{M}$ ) with an incubation time of 30 min. The time course experiments were carried out over 0 to 24 h with 750 : 750  $\mu\text{M}$  t-BHP :  $\text{FeSO}_4$  final oxidant mixture concentration. The 0 h experiment involved the rapid addition of the oxidant mixture to the cell suspension and centrifuging immediately afterwards.

All treatments were terminated by centrifugation at 4000 g for 5 min at 4°C after the transfer of cell suspensions into 1.5 ml eppendorf tubes. Thereafter, supernatants were taken for LDH analysis.

#### 2.2.6.2 *With Organic solvents*

Four organic solvents, all HPLC – grade (methanol, ethanol, chloroform and hexane) were tested for their toxicities in U937 cells. These solvents were sterile-filtered using Corning nylon, sterile, syringe filters (0.22  $\mu\text{m}$ ). A serial dilution from 0.2% to 1.0% (v/v) was carried out aseptically in CM by the addition of 2 to 10  $\mu\text{l}$  of solvents. 0.9 ml of CM containing the solvents to be tested was added to each well of a 24-well plate already containing 0.1 ml of cell suspension. Each well contained  $0.5 \times 10^6$  cells/ml, all from the same population. The final solvent concentration varied from 0.2% to 1.0% (v/v). In all experiments, controls contained cells in CM only without the solvent of interest. Cells were incubated for 24 h at 37°C and 5%  $\text{CO}_2$  in a humid environment. All treatments were stopped by centrifuging at 250 g for 5 min at 4°C, prior to LDH analysis.

### 2.2.6.3 With fatty acids

Fatty acids (FAs) were complexed to fatty-acid-free bovine serum albumin (BSA) aseptically at a molar ratio of 2.5: 1 before CM supplementation. Fatty acid (FA) solutions in EtOH ranging from 2.5 to 10 mM were prepared from a 100 mM stock solution and stored under nitrogen after sterile filtration using nylon syringe filters. FA : BSA complexes were prepared by the addition of an equal volume of 2.5 – 10 mM of FA to 1 – 4 mM of BSA respectively and further diluted with CM to a final volume of 0.9 ml. CM supplemented with FA (0.9 ml) was then added to 0.1 ml of cell suspension in each well of a 24-well plate. Untreated experiments involved cells in CM only and “vehicle only” controls, cells in CM with 0.4% (v/v) ethanol. Each well contained  $0.5 \times 10^6$  cells/ml. Cells were incubated for 24 h at 37°C and 5% CO<sub>2</sub> in a humid environment. Final fatty acid concentration ranged from 10 to 40  $\mu$ M. The EtOH concentration in the culture medium was 0.4% (v/v), which had no effect on cell viability as measured by LDH analysis (2.3.5.3).

Cells were incubated with 10  $\mu$ M of individual fatty acids (SA, OA, AA, EPA, DHA) complexed with fatty-acid-free BSA in the molar ratio 2.5 : 1 using equal volumes of 10 mM FA and 4 mM BSA. In controls, EtOH instead of FA was complexed with BSA. Cells were incubated in 4 ml suspensions at  $0.5 \times 10^6$  cells/ml in 25 cm<sup>2</sup> culture flasks. All flasks were loosely capped and incubated at 37°C and 5% CO<sub>2</sub> in a humid environment for 3 d (72 h).

Cells were also incubated with 40  $\mu$ M of each fatty acid (SA, OA, AA, EPA or DHA) or ethanol only (control) complexed to BSA at a molar ratio 2.5: 1 without medium change for 72 hours. Incubations were performed in 4 ml cell suspensions at  $0.5 \times 10^6$  cells/ml in 25 cm<sup>2</sup> culture flasks. After 3 days, cells were washed twice in HBSS and

cell densities re-adjusted to contain  $0.5 \times 10^6$  cells/ml. Cell suspensions were centrifuged at 250 g for 5 min at 4 °C, supernatant discarded and cells re-suspended in freshly prepared CM without fatty acid or ethanol (control) supplementation for a further 3 days.

For all experiments, reactions were terminated by centrifuging at 250 g for 5 min at 4°C, prior to LDH analysis.

#### **2.2.6.4 With antioxidants**

##### **(A) Ascorbic acid**

A 100 mM stock solution of ascorbic acid was prepared in nano-pure water, sterile filtered using polyethersulfone, 0.2  $\mu\text{m}$  – pore size Corning filters and stored at 2 – 8°C for a month. By serial dilution, 4 – 20 mM ascorbic acid solutions were prepared from 100 mM stock solutions just before use. CM supplemented with ascorbic acid to a final volume of 0.9 ml was added to each well of a 24-well plate containing 0.1 ml of cell suspension. Final concentration of ascorbic acid varied from 0 mM to 500 mM. Each well contained  $0.5 \times 10^6$  cells/ml. Controls were incubated with CM only. All reactions were stopped by centrifuging at 250 g for 5 min at 4°C and supernatants taken for LDH analysis.

##### **(B) $\alpha$ -tocopherol**

A stock solution of  $\alpha$ -tocopherol was prepared at 100 mM in ethanol and stored under nitrogen at 2 – 8°C for a month. 10 – 50 mM  $\alpha$ -tocopherol in ethanol was prepared by serial dilution from 100 mM stock solution and diluted in the culture medium to obtain 10 – 40  $\mu\text{M}$  final concentrations in 1 ml cell suspensions. 0.9 ml of CM supplemented with  $\alpha$ -tocopherol was added to each well of a 24-well plate containing 0.1 ml of cell

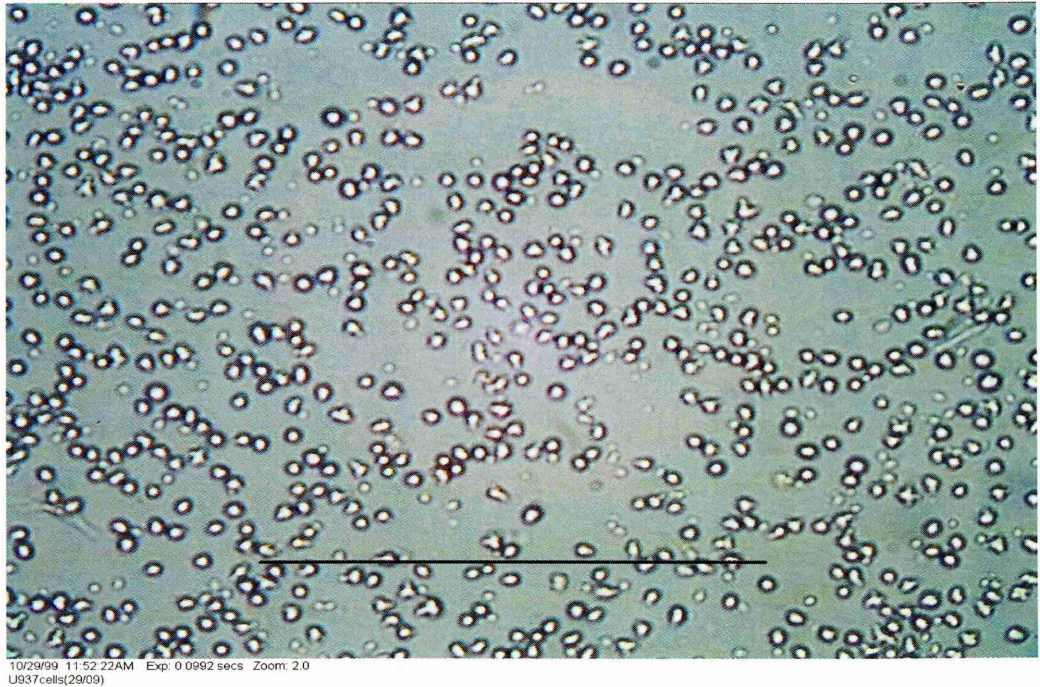
suspension. Each well contained  $0.5 \times 10^6$  cells/ml. For controls, cells were incubated with CM with 0.1% (v/v) of ethanol only. Final ethanol concentration in cell suspensions was 0.1% (v/v) in all wells. All reactions were stopped by centrifuging at 250 g for 5 min at 4°C before LDH analysis.

**(C) *β-carotene/ Astaxanthin***

A 100 mM stock solution of β-carotene or astaxanthin was prepared in chloroform, sterile-filtered using a Corning nylon sterile syringe filter, 0.2 μm pore size and stored under nitrogen at – 20°C for a month. 3 mM – 12 mM β-carotene or astaxanthin was prepared in CHCl<sub>3</sub> by serial dilution from the 100 mM stock solution. CM supplemented with aliquots of 3 – 12 mM β-carotene or astaxanthin (0.9 ml final volume) was sonicated for 5 s in an ice bath and sterile – filtered using nylon syringe filters. 0.9 ml of carotenoid supplemented CM, was added to each of a 24-well plate containing 0.1 ml of cell suspension. Final concentration of β-carotene or astaxanthin varied from 0 to 12 μM. Each well contained  $0.5 \times 10^6$  cells/ml. Untreated cells involved incubations in CM only and “vehicle only” controls containing 0.1% (v/v) of chloroform. The CHCl<sub>3</sub> concentration in the culture medium in all experiments was 0.1% (v/v).

## 2.3 RESULTS

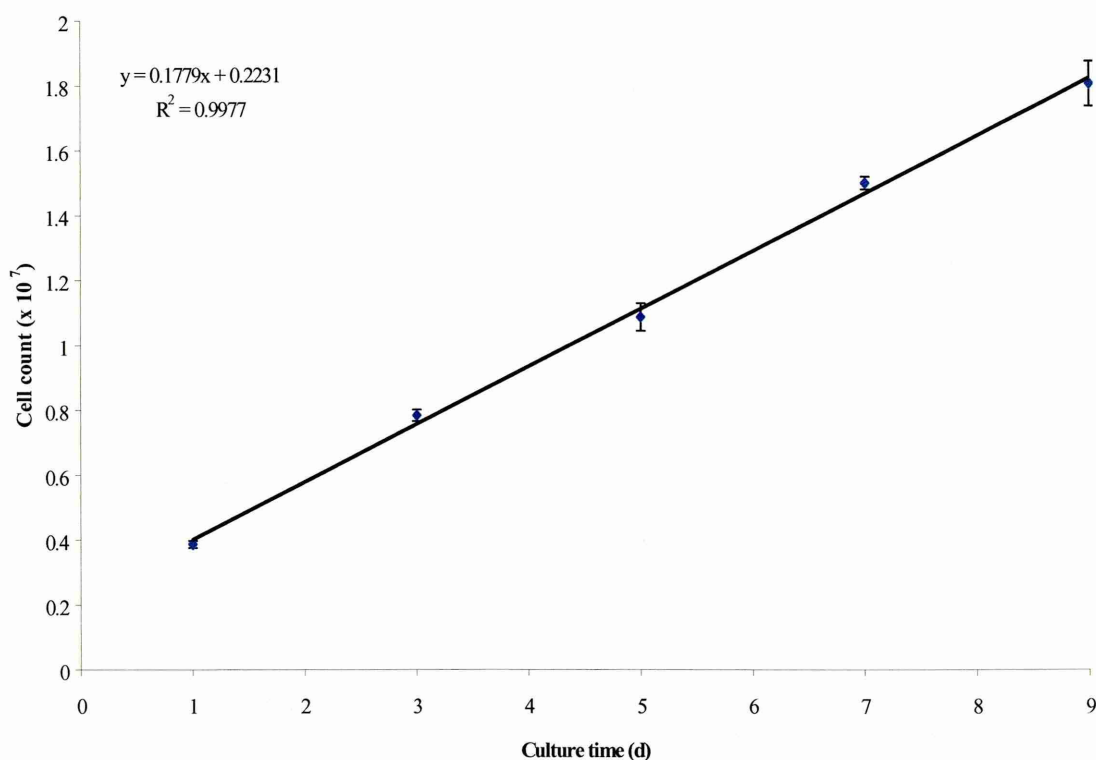
### 2.3.1 U937 cells



**Fig. 2.1** U937 cells in culture (x 2000 magnification). Scale bar represents 100  $\mu$ m.

### 2.3.2 Cell growth of U937 cells

U937 cells were grown over 9 days at 37°C and 5% CO<sub>2</sub> in a humid environment in a CO<sub>2</sub> incubator. Cells were counted on day 1, 3, 5, 7 and 9 immediately after thawing. Frozen cells were thawed from LN<sub>2</sub> and established in culture as earlier described in sections 2.2.3.1 and 2.2.3.2. Under these conditions, U937 cells grew in a linear fashion (Fig. 2.2). Cells were initially grown in 25 cm<sup>2</sup> culture flasks on day 0 of thawing. 10 ml of freshly prepared culture medium (CM) was added to cells in 75 cm<sup>2</sup> culture flasks on day 1 of thawing giving a final volume of 20 ml that was maintained in all flasks throughout the course of the experiment.



**Fig. 2.2** Continuous U937 cell culture over 9 days after thawing from LN<sub>2</sub>. Results show means  $\pm$  95% confidence intervals (CIs) of counts from triplicate flasks performed in duplicates. Correlation coefficient ( $R^2$ ) = 0.9977;  $P < 0.05$ ;  $DF = 1$ . Cell growth was not extended over 9 days because flasks need to be split after the ninth day for optimal cell growth and viability.

### 2.3.3 Cell viability monitoring

Cells were recovered from LN<sub>2</sub> at viability  $> 90\%$  (Table 2.2) and a high viability maintained for 7 days. On day 9 however, viability significantly decreases below 90% due to the cell density. As the cells become over-crowded, they tend to encroach on each other, thus reducing cell viability as shown in Table 2.2.

**Table 2.2** Viability of U937 cells over 9 days. Means  $\pm$  95% CIs from duplicate experiments (n = 3).

Culture time (d)	Viability (Fraction)	Cells/ml ( $\times 10^6$ )
Day 1	$0.9195 \pm 0.0128$	$0.3861 \pm 0.0111$
Day 3	$0.9269 \pm 0.0175$	$0.3917 \pm 0.0086$
Day 5	$0.9330 \pm 0.0137$	$0.5433 \pm 0.0214$
Day 7	$0.9396 \pm 0.0157$	$0.7500 \pm 0.0098$
Day 9	$0.8823 \pm 0.0034$	$0.9033 \pm 0.0346$

Cell viability on day 9 differed significantly from days 1, 3, 5 and 7 when Tukey's pairwise comparisons was carried out post analysis of variance (One-Way ANOVA  $F = 10.64$ ,  $P < 0.01$ ,  $DF = 4$ ).

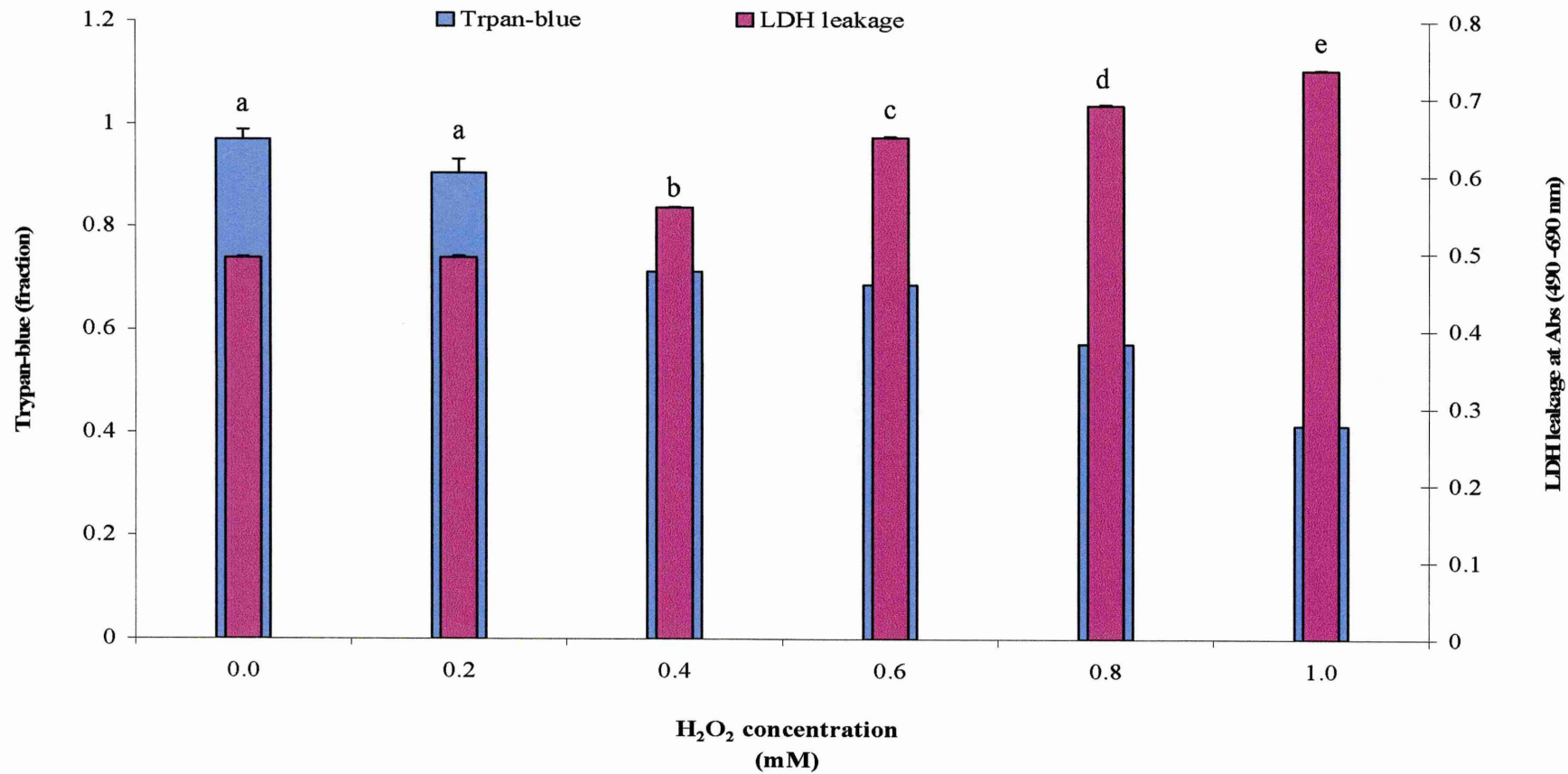
#### 2.3.4 Comparison of Trypan Blue and LDH methods of viability measurements

To investigate the relationship between Trypan Blue and LDH activity methods of cell viability measurements, U937 cells were incubated with hydrogen peroxide at concentrations ranging from 0 – 1 mM for 24 h. Thereafter, the cells were counted by dye-exclusion and the extent of membrane leakage established by measuring LDH in cell supernatants. There was a dose-dependent decrease in cell viability by Trypan Blue and an increase in LDH leakage with increasing  $H_2O_2$  concentrations when compared to controls (Fig.2.3). The Trypan Blue method is invasive, laborious, time-consuming and prone to bias by the naked eye. On the contrary, the LDH assay is non-invasive, rapid and to a great extent, eliminates operator errors which may occur during sampling, dilution, mixing, chamber filling and inaccurate counting with the Trypan Blue method.



U937 cells were subjected to oxidative stress with 0 – 1 mM H<sub>2</sub>O<sub>2</sub> for 24 h prior to Trypan Blue and LDH analysis. Fig. 2.3, shows Trypan Blue results calculated as a fraction of the total cell count and LDH results were expressed as Absorbance at (490 – 690) nm (2.2.4.2). When a cell suspension is stained with Trypan Blue, viable cells stay small, round and refractile whereas non-viable or dead cells become swollen, larger and dark blue enabling the determination of both the total count of cells per ml and percentage of viable cells. Cells were counted within 2 minutes of staining with the dye. The higher the fraction obtained for viable cells the healthier the cell suspension. On the other hand, the LDH assay assumes that LDH leaks after damage to the cell membrane and that all activity is rapidly released from damaged cells into the surrounding environment. Hence, the higher the rate of leakage of LDH activity, the greater the extent of cell damage or death i.e. cell suspensions with higher enzyme activity have been exposed to more membrane damage.

For all treatments, parameters such as pH, dissolved oxygen concentration, medium composition and culture age that may modulate LDH release rate were kept constant.



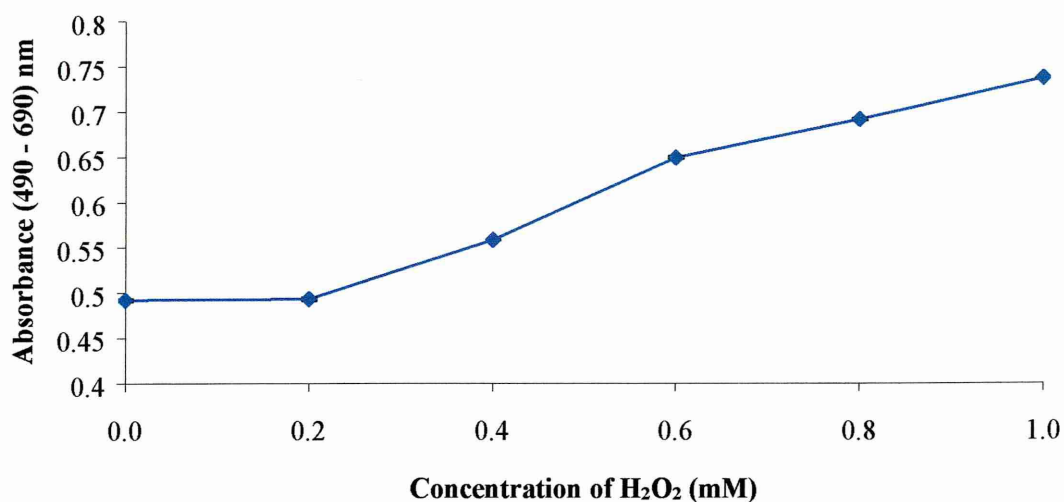
**Fig. 2.3** Comparison between the Trypan Blue and LDH leakage viability measurements after 24 h of incubation with 0 – 1 mM  $H_2O_2$ . The values represent means  $\pm$  95% CIs of 5 replicate wells (n = 5). Letters: a, b, c, d and e indicate significant differences between means at  $P < 0.01$ .

Both methods of viability measurements showed similar results (Fig. 2.3) bearing in mind that the higher Trypan Blue levels for viable cells produce lower LDH activity. A correlation of LDH absorbance values with increasing  $\text{H}_2\text{O}_2$  concentrations gave a better correlation ( $R^2 = 0.98$ ,  $P < 0.01$ ;  $N = 5$ ) than Trypan Blue values correlated with  $\text{H}_2\text{O}_2$  concentrations ( $R^2 = 0.962$ ,  $P < 0.01$ ;  $N = 5$ ), although the difference was not significant. This study shows a high correlation between both methods however, the LDH activity method was chosen for routine viability determinations because it gave a higher correlation with increasing toxicity and it also limits operator errors and bias.

### **2.3.5 LDH activity results**

#### ***2.3.5.1 Effects of hydrogen peroxide on LDH in U937 cells***

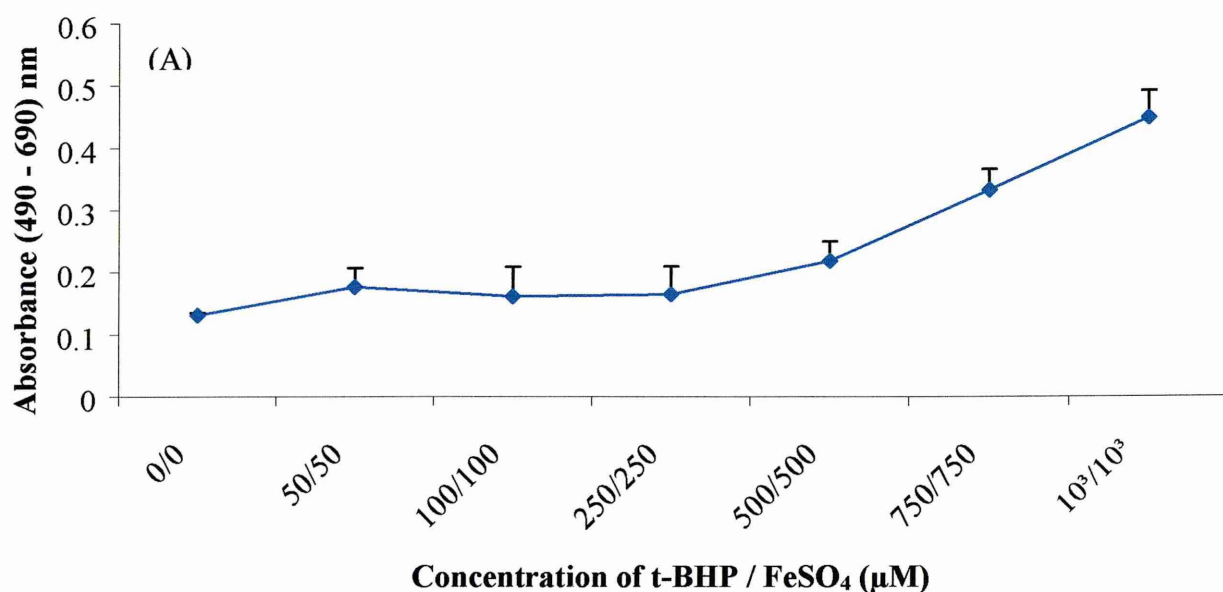
Cells were incubated for 24 h in supplemented CM supplemented with 0 to 1 mM  $\text{H}_2\text{O}_2$  (Fig. 2.4). LDH increased significantly with increasing  $\text{H}_2\text{O}_2$  concentration (One-Way ANOVA  $F = 2.8 \times 10^4$ ,  $P < 0.01$ ,  $DF = 5$ ). Tukey's pairwise comparisons showed a significant difference from controls at concentrations  $\geq 0.4$  mM. There was no difference however at  $\text{H}_2\text{O}_2$  concentration of 0.2 mM compared to controls. There was a linear LDH increase with increasing  $\text{H}_2\text{O}_2$  levels ( $R^2 = 0.959$ ).

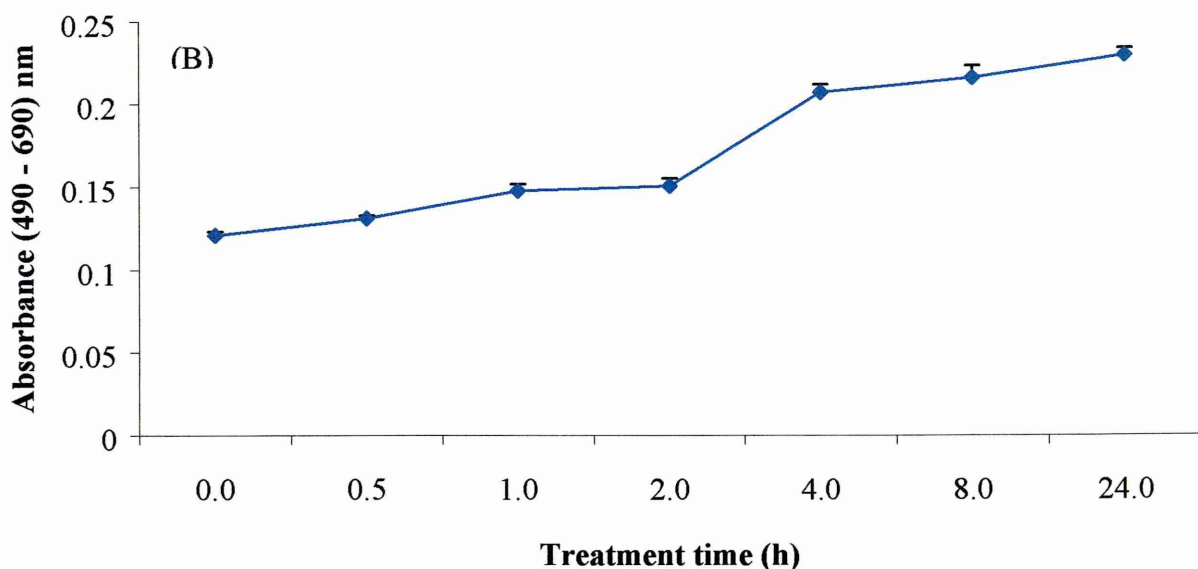


**Fig. 2.4** LDH leakage from the incubation of U937 cells with 0 – 1 mM H<sub>2</sub>O<sub>2</sub> for 24 h. Means  $\pm$  95% CIs (n = 5).

#### 2.3.5.2 Effects of oxidative stress with t-BHP : FeSO<sub>4</sub> on U937 model systems

In order to determine the effects of oxidative stress on U937 cell membrane integrity, cells were treated with a mixture of t-BHP and FeSO<sub>4</sub>. Fig. 2.5 (A) shows the results for cells treated with varying concentrations of the radical oxidizing system of t-BHP and Fe<sup>2+</sup> for 30 min.





**Fig. 2.5** U937 cells were incubated with various concentrations of the radical oxidizing system (t-BHP:FeSO<sub>4</sub>). Cell viability was expressed as absorbance values (490 – 690) nm. Points represent means  $\pm$  95% CIs of cells from 4 replicate wells. Dose response of cells to oxidant mixture after 30 min of incubation (A), and time dependent response of cells to 750 : 750  $\mu$ M of t-BHP : FeSO<sub>4</sub> oxidant mixture over 24 h (B) at 37°C and 5% CO<sub>2</sub> in a humid environment (n = 4).

There was a concentration – dependent increase in membrane leakage of LDH (Kruskall-wallis  $H = 22.91$ ,  $P < 0.01$ ,  $DF = 6$ ). Dunn’s test showed a significant difference in LDH released into the culture medium at concentrations  $\geq 750 : 750 \mu$ M for 30 min compared to controls.

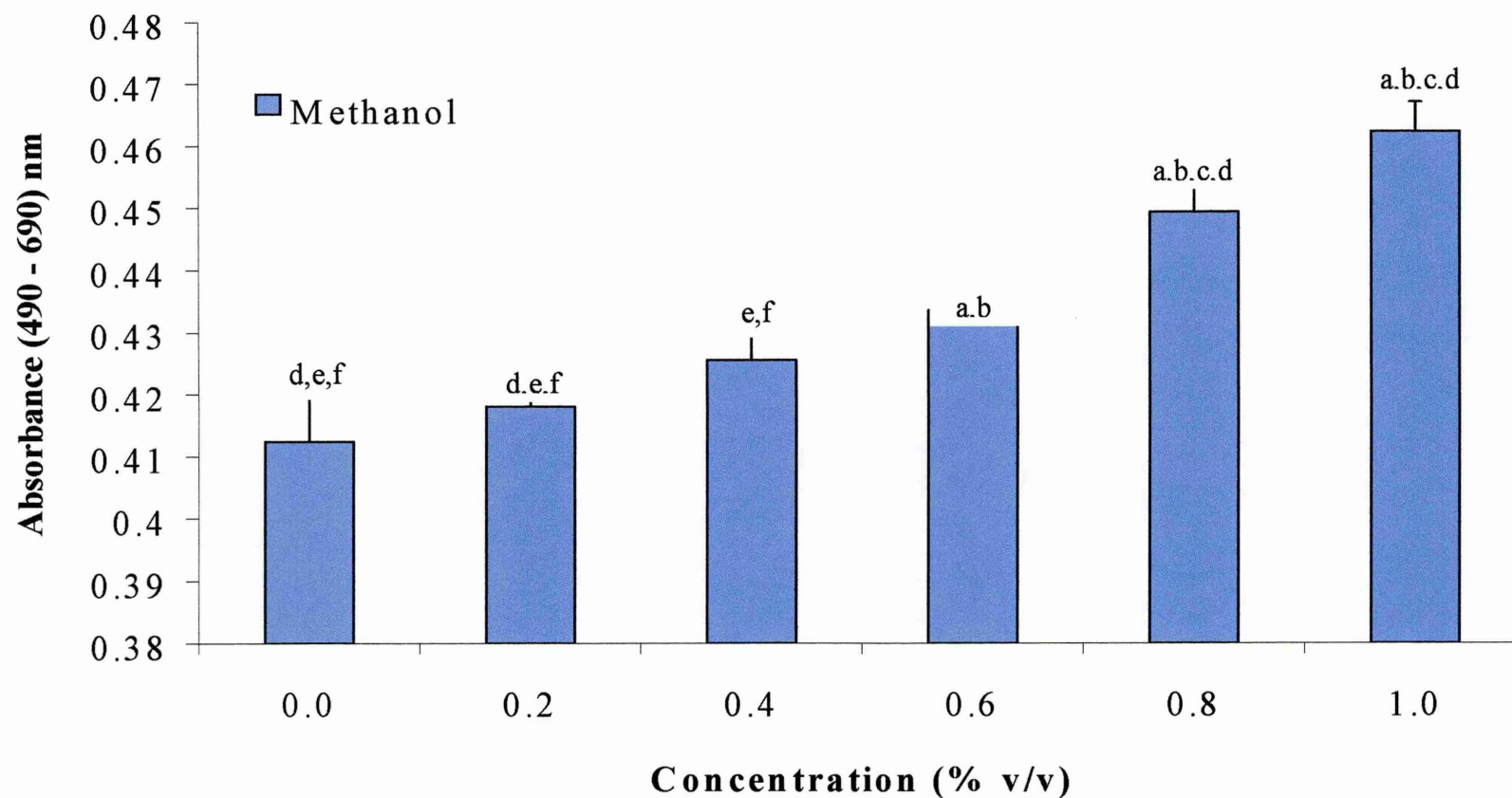
The effect of treatment with 750  $\mu$ M t-BHP and 750  $\mu$ M FeSO<sub>4</sub> over a time course of 0 – 24 h was also investigated and the LDH values are as shown in Fig. 2.5 B. Tukey’s pairwise comparison shows a significant loss of membrane integrity after 30 min of exposure compared to the 0 h control (one-way ANOVA  $F = 403.84$ ,  $P < 0.01$ ;  $N = 4$ ).

A correlation of absorbance with time showed a significant linear relationship between the variables ( $R^2 = 0.961$ ;  $P < 0.01$ ;  $N = 4$ ) (Fig. 2.5 B).

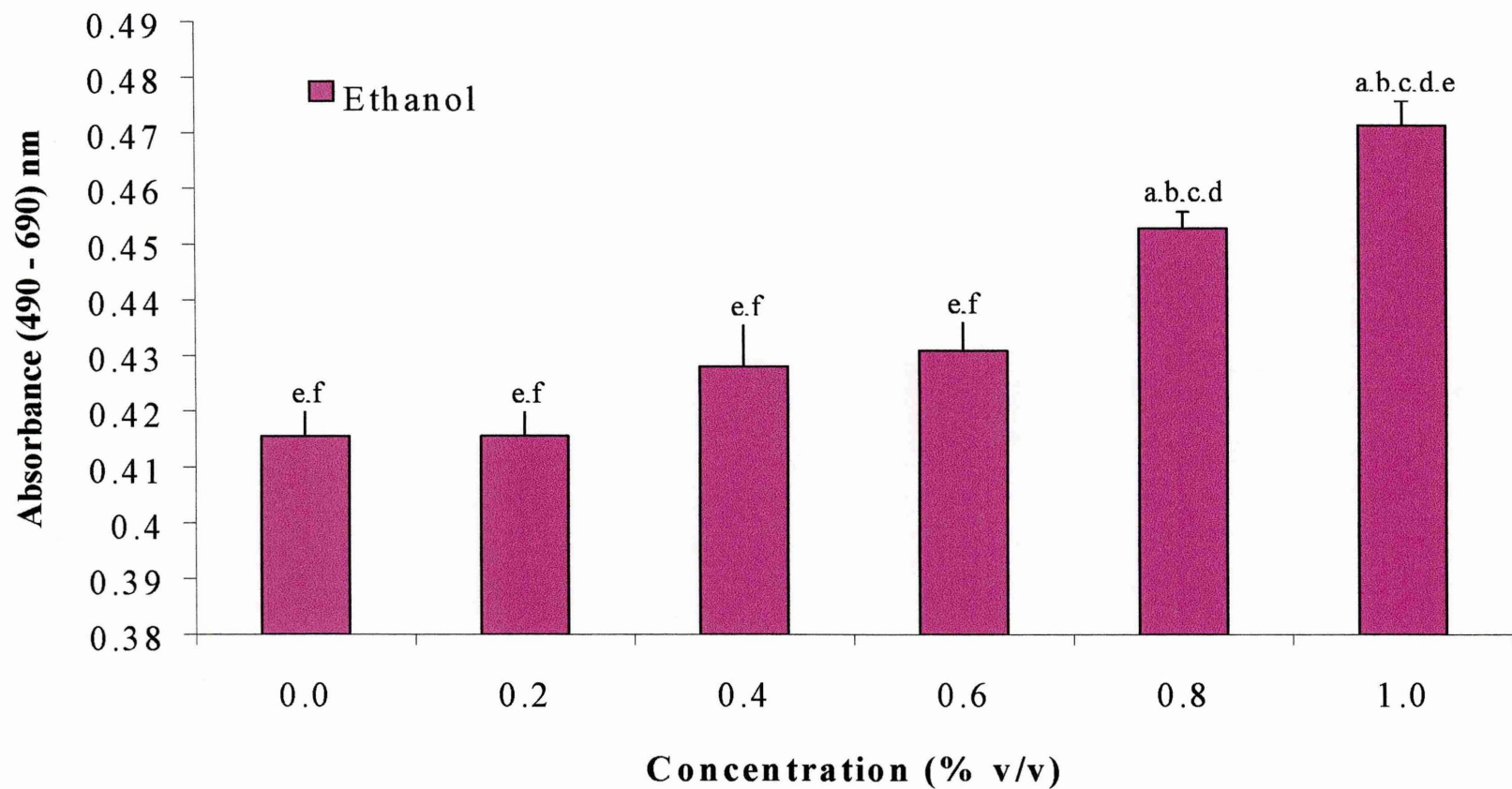
For the dose-dependent experiment, LDH leakage was negligible at concentrations  $\leq 500 : 500 \mu\text{M}$  oxidant levels. However at 750 and 1000  $\mu\text{M}$  oxidant mixture concentrations, a significant increasing membrane leakage was observed. There was a 154% and 242% increase at 750 and 1000  $\mu\text{M}$  oxidant concentration respectively compared to control values. After the treatment of cells with 750 : 750  $\mu\text{M}$  oxidant mixture, LDH activity after  $\frac{1}{2}$ , 1, 2, 4, 8 and 24 h was 8, 22, 25, 71, 78 and 89% respectively above the control.

#### **2.3.5.3 With different organic solvents**

Cells exposed to methanol, ethanol, hexane and chloroform in concentrations ranging from 0.2% to 1.0% (v/v) were analysed for LDH leakage into the medium as a measure of cell membrane integrity loss associated with toxicity. This study was conducted to determine the tolerance levels of U937 cells to these organic solvents, which will later be used to dissolve certain water-insoluble agents. Of the solvents tested, only chloroform had no significant effect on cell viability (Fig. 2.8) all the others were toxic to cells at different solvent concentrations. After Tukey's pairwise comparisons, methanol (Fig. 2.6) showed a significant increase in LDH release at concentrations  $\geq 0.6\%$  (v/v) (one-way ANOVA  $F = 34.27$ ,  $P < 0.01$ ,  $N = 4$ ); ethanol (Fig. 2.7), at levels  $\geq 0.8\%$  (v/v) (one-way ANOVA  $F = 38.61$ ,  $P < 0.01$ ,  $N = 4$ ); hexane (Fig. 2.9), had significant LDH leakage in cells incubated with  $\geq 0.6\%$  (v/v) (one-way ANOVA  $F = 32.14$ ,  $P < 0.01$ ,  $N = 4$ ) whereas, cells treated with chloroform showed no significant loss of membrane integrity at these concentrations (one-way ANOVA  $F = 1.12$ ,  $P > 0.05$ ,  $N = 4$ ). All observations were made after 24 h.

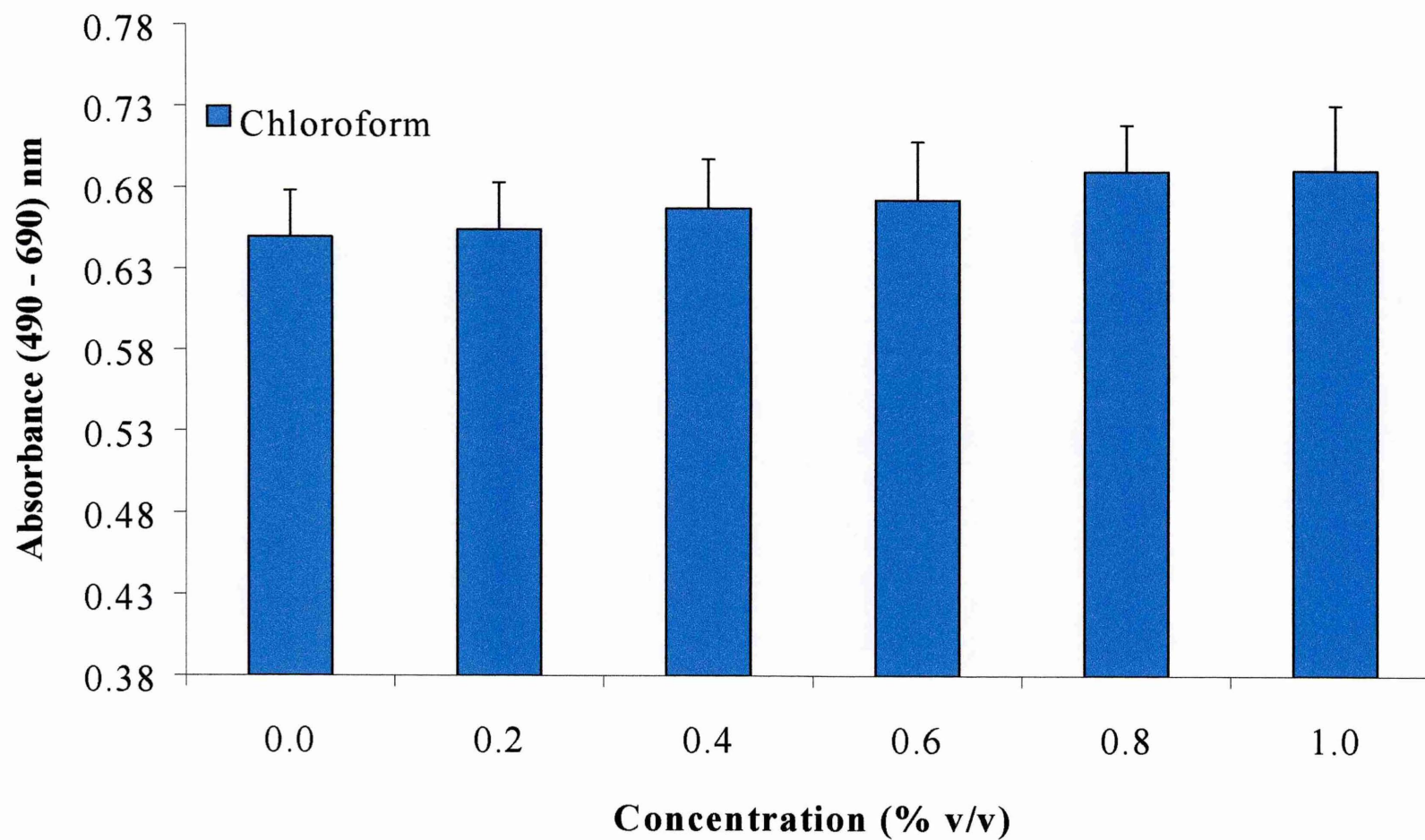


**Fig. 2.6** LDH release after 24 h of incubation with 0 – 1% (v/v) methanol. a: Indicates significant difference from solvent-untreated controls (0% v/v); b: 0.2% v/v; c: 0.4% v/v; d: 0.6% v/v; e: 0.8% v/v and f: 1.0% v/v by one-way ANOVA at  $P < 0.01$ . Values represent means  $\pm$  95% CIs of duplicate experiments ( $n = 4$ ).

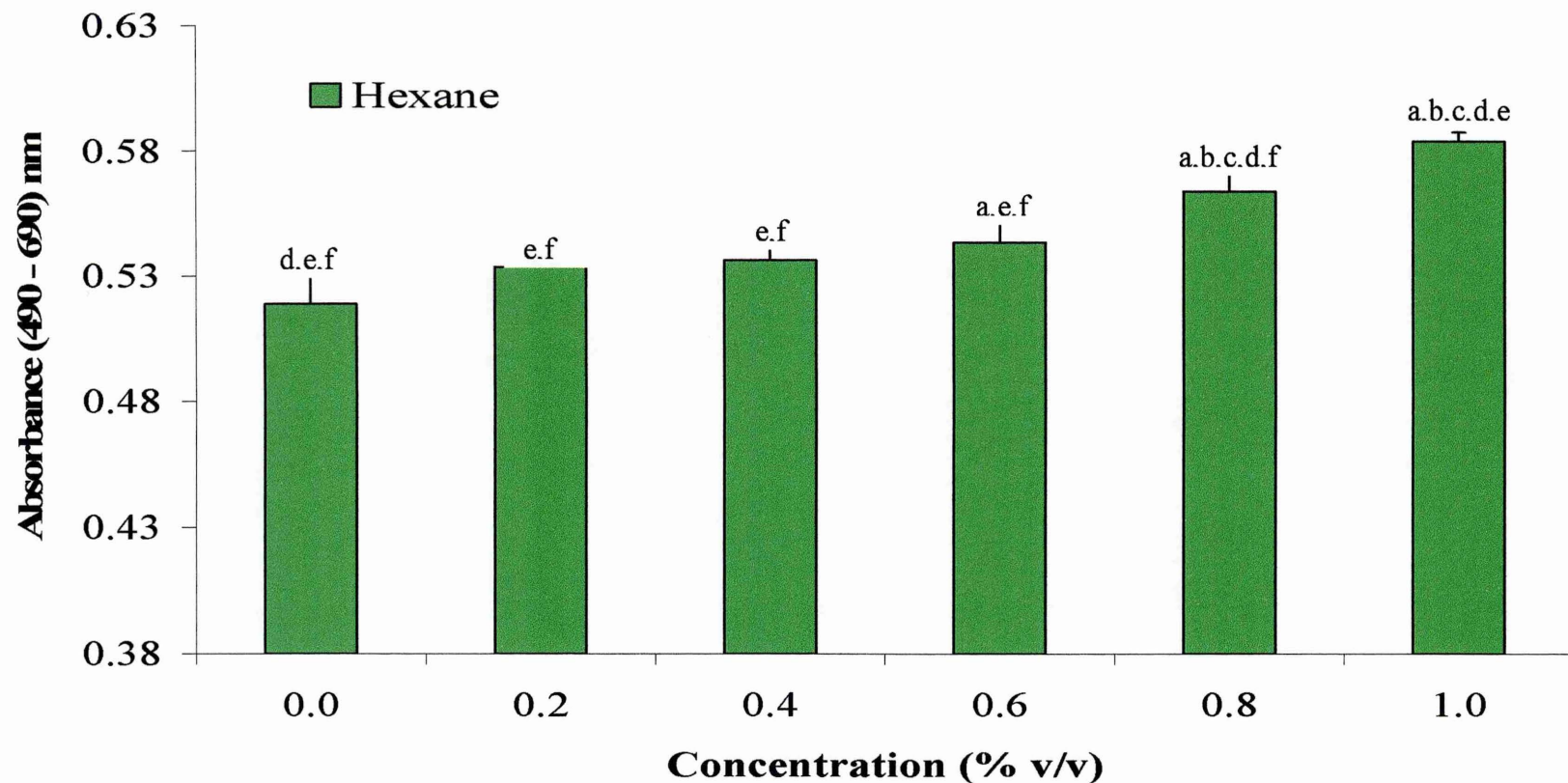


**Fig. 2.7** LDH release after 24 h of incubation with 0 – 1% (v/v) ethanol. a: Represents significant difference from solvent-untreated controls (0% v/v); b: 0.2% v/v; c: 0.4% v/v; d: 0.6% v/v; e: 0.8% v/v and f: 1.0% v/v (one-way ANOVA,  $P < 0.01$ ). Means  $\pm$  95% CIs of two independent experiments ( $n = 4$ ).





**Fig. 2.8** LDH release after 24 h of incubation with 0 – 1% (v/v) chloroform. Means  $\pm$  95% CIs of two separate experiments (n = 4).



**Fig. 2.9** LDH release after 24 h of incubation with 0 – 1% (v/v) hexane. a: Denotes significant difference from solvent-untreated controls (0% v/v); b: 0.2% v/v; c: 0.4% v/v; d: 0.6% v/v; e: 0.8% v/v and f: 1.0% v/v by one-way ANOVA at  $P < 0.01$ . Means  $\pm$  95% CIs of two separate experiments ( $n = 4$ ).

#### 2.3.5.4 With fatty acids

To investigate the possible toxic effects of fatty acids on cell viability, U937 cells were incubated with 0 – 40  $\mu$ M of the saturated fatty acid SA (18:0), the monounsaturated fatty acid OA (18:1), or the very long chain PUFAs AA (20:4n-6), EPA (20:5n-3) and DHA (22:6n-3) all complexed with BSA for 24 h after which the extent of membrane leakage was determined. Tables 2.3 and 2.4 shows absorbance values after the treatment of cells with SA, OA, AA, EPA and DHA for 24 h compared to controls.

**Table 2.3** Effects of SA, OA and AA on LDH leakage after 24 h of incubation. Values represent means  $\pm$  95 CIs from two independent experiments (n = 4). “No treatment” represents cells in CM10 only and “vehicle only”, cells treated with 0.4% v/v of ethanol.

Concentration	SA	OA	AA
No treatment	0.1569 $\pm$ 0.0014	0.1630 $\pm$ 0.0012	0.2197 $\pm$ 0.0053
Vehicle only	0.1554 $\pm$ 0.0101	0.1637 $\pm$ 0.0047	0.2289 $\pm$ 0.0057
10 $\mu$ M	0.1573 $\pm$ 0.0107	0.1638 $\pm$ 0.0047	0.2255 $\pm$ 0.0041
20 $\mu$ M	0.1622 $\pm$ 0.0123	0.1653 $\pm$ 0.0029	0.2311 $\pm$ 0.0116
30 $\mu$ M	0.1668 $\pm$ 0.0159	0.1658 $\pm$ 0.0048	0.2211 $\pm$ 0.0162
40 $\mu$ M	0.1661 $\pm$ 0.0137	0.1667 $\pm$ 0.0026	0.2345 $\pm$ 0.0134

**Table 2.4** Effects of EPA and DHA on LDH leakage after 24 h of incubation.

Values represent means  $\pm$  95 CIs from two independent experiments ( $n = 4$ ). “No treatment” represents cells in CM10 only and “vehicle only”, cells treated with ethanol (0.4% v/v).

Concentration	EPA	DHA
No treatment	$0.1449 \pm 0.0033$	$0.2446 \pm 0.0103$
Vehicle only	$0.1460 \pm 0.0019$	$0.2260 \pm 0.0065$
10 $\mu$ M	$0.1450 \pm 0.0042$	$0.2312 \pm 0.0111$
20 $\mu$ M	$0.1506 \pm 0.0053$	$0.2383 \pm 0.0114$
30 $\mu$ M	$0.1513 \pm 0.0020$	$0.2383 \pm 0.0057$
40 $\mu$ M	$0.1533 \pm 0.0049$	$0.2492 \pm 0.0164$

Neither SA, OA, AA, EPA nor DHA showed significant effects on LDH leakage into culture supernatant after 24 h of treatment with 0 – 40  $\mu$ M complexed with BSA in a 2.5: 1 molar ratio when compared to untreated cells (No treatment). For SA (one-way ANOVA  $F = 0.68$ ,  $P > 0.05$ ,  $DF = 5$ ); OA (one-way ANOVA  $F = 0.56$ ,  $P > 0.05$ ,  $DF = 5$ ); AA (one-way ANOVA  $F = 1.15$ ,  $P > 0.05$ ,  $DF = 5$ ); EPA (one-way ANOVA  $F = 2.72$ ,  $P > 0.05$ ,  $DF = 5$ ) and DHA (one-way ANOVA  $F = 2.36$ ,  $P > 0.05$ ,  $DF = 5$ ). This study shows no marked increase in LDH release at FA concentrations ranging from 0 to 40  $\mu$ M when complexed with BSA in U937 cells after 24 h incubation. See appendix for reasons for the choosing certain statistical methods of analysis over others.

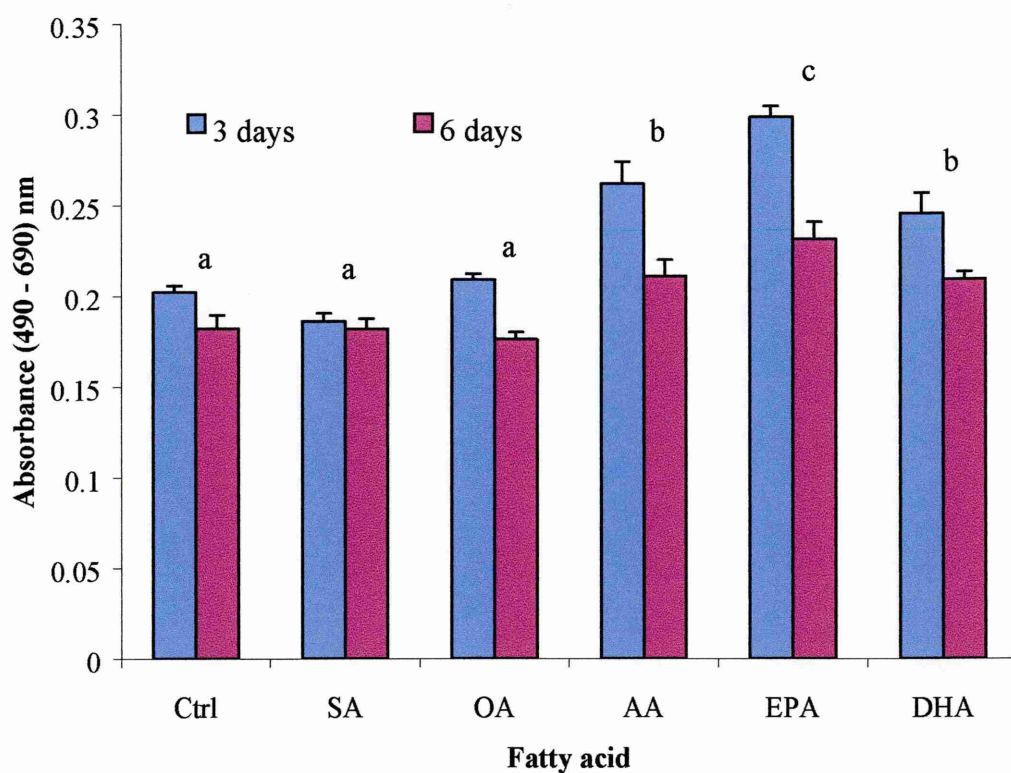
To determine the effect of prolonged treatment of cells with fatty acids, cells were incubated with 10  $\mu$ M SA, OA, AA, EPA and DHA for 3 days. Results showed no significant effects on LDH release after extended treatments with these fatty acids for 3 days (one-way ANOVA  $F = 1.57$ ,  $P > 0.05$ ,  $DF = 5$ ,  $N = 4$ ) (Table 2.5).

**Table 2.5** LDH leakage in U937 cells incubated with 10  $\mu$ M SA, OA, AA, EPA and DHA for 72 h.

Means  $\pm$  95% CIs for duplicate experiments (n = 4).

Fatty Acid	LDH Absorbance values
Control (Ctrl)	0.2180 $\pm$ 0.0079
Stearic Acid (SA)	0.2153 $\pm$ 0.0040
Oleic Acid (OA)	0.2201 $\pm$ 0.0050
Arachidonic Acid (AA)	0.2236 $\pm$ 0.0045
Eicosapentaenoic Acid (EPA)	0.2234 $\pm$ 0.0033
Docosahexaenoic Acid (DHA)	0.2220 $\pm$ 0.00049

The effect of 40  $\mu$ M FA on cells was investigated over a prolonged period (72 h), a concentration that was otherwise non-toxic to U937 cells after 24h of incubation. Cells incubated with the PUFAs showed a higher LDH leakage than control cells. Multiple comparisons of LDH results after 72 h with 40  $\mu$ M levels of the five different fatty acids with control, showed a significant difference between treatments (One-way ANOVA  $F = 117.65$ ,  $P < 0.01$ ,  $DF = 5$ ). Tukey's pairwise comparison showed no significant difference between the saturated fatty acid (SA), the monounsaturated fatty acid (OA) compared to controls. However, there was a significant increase in LDH levels with the three polyunsaturated fatty acids (AA, EPA and DHA) compared to controls. The highest LDH leakage was however, surprisingly obtained with EPA treatment which, was found to be significantly higher than that quantified with AA and even DHA. Washing with HBSS and re-suspension of cells in freshly prepared CM, with fatty acid withdrawal and incubation for a further 3 days showed no reversal of these toxic effects. Tukey's pairwise comparisons (one-way ANOVA  $F = 38.41$ ,  $P < 0.01$ ,  $DF = 5$ ) (Fig. 2.10).



**Fig. 2.10** LDH effects after three days of FA supplementation (3 d) and three more days in the absence of these fatty acids (6 d). Means  $\pm$  95% CIs ( $n = 4$ ). a, b and c represent significant differences between means ( $P < 0.01$ ).

To determine if the membrane leakage observed in U937 cells after 72 h incubation with 40  $\mu$ M AA, EPA and DHA could be reversed, cells were washed, diluted to a density of 500000 cells/ml and re-established in culture with fatty-acid-free CM. Though the effects were not reversed, there was a significant decrease in the extent of membrane leakage observed after six days when compared to controls (ANOVA;  $F = 36.35$ ,  $P < 0.01$ ,  $DF = 1$ ). When compared to controls, there was a 47, 44 and 31% decrease in LDH leakage with AA, EPA and DHA treatments respectively, after re-incubation in a fatty-acid-free medium.

### 2.3.5.5 Treatment of cells with Antioxidants

#### (A) Ascorbic acid

To examine the effect of ascorbic acid on U937 cell membrane integrity, cells were incubated with 0 – 500  $\mu$ M ascorbic acid for 24 h and the LDH leakage into culture medium was measured. LDH leakage was negligible at these ascorbic acid concentrations (Table 2.6).

**Table 2.6** LDH activity with increasing ascorbic acid concentration. U937 cells (500000 cells/ml) were exposed to 0 – 500  $\mu$ M ascorbic acid for 24 h in culture medium. Absorbance readings represent means  $\pm$  95% CIs for two independent experiments (n = 4).

Ascorbic acid concentration	Absorbance (490 – 690) nm
0 $\mu$ M	0.4764 $\pm$ 0.0053
100 $\mu$ M	0.4752 $\pm$ 0.0057
200 $\mu$ M	0.4750 $\pm$ 0.0059
300 $\mu$ M	0.4794 $\pm$ 0.0057
400 $\mu$ M	0.4870 $\pm$ 0.0034
500 $\mu$ M	0.4919 $\pm$ 0.0183

There was no significant increase in the loss of membrane integrity with ascorbic acid concentrations ranging from 0 – 500  $\mu$ M (One-way ANOVA F = 2.42, P > 0.05, DF = 5, N = 4).

**(B) *α-tocopherol***

CM was supplemented with 0 – 50  $\mu\text{M}$   $\alpha$ -tocopherol with ethanol as the vehicle at 0.1% (v/v) final ethanol concentration (1  $\mu\text{l/ml}$ ) for 24 h (Table 2.7). Thereafter, LDH activity was determined.

**Table 2.7** Effects of  $\alpha$ -tocopherol on LDH activity in U937 cells after 24 h of incubation. Means  $\pm$  95% CIs of two independent experiments (n = 4).

$\alpha$ -tocopherol concentration	Absorbance (490 – 690) nm
0 $\mu\text{M}$	0.3888 $\pm$ 0.0139
10 $\mu\text{M}$	0.3897 $\pm$ 0.0141
20 $\mu\text{M}$	0.3836 $\pm$ 0.0054
30 $\mu\text{M}$	0.3845 $\pm$ 0.0083
40 $\mu\text{M}$	0.3923 $\pm$ 0.0040
50 $\mu\text{M}$	0.4077 $\pm$ 0.0150

One-way ANOVA showed no significant difference in LDH activity between treatments ( $F = 2.43$ ,  $P > 0.05$ ,  $DF = 5$ ,  $N = 4$ ) when compared to controls (0.1% v/v ethanol only) (Table 2.7).

**(C) *With carotenoids***

To check the toxic levels of  $\beta$ -carotene and astaxanthin in U937 cells by measuring LDH leakage from membranes, cells were incubated with 0 – 12  $\mu\text{M}$   $\beta$ -carotene and astaxanthin using 0.1% (v/v) of chloroform as the vehicle of transport.



**Table 2.8** LDH effects with  $\beta$ -carotene and astaxanthin after 24 h of incubation. Absorbance values represent means  $\pm$  95% CIs of two independent experiments (n = 4). “No treatment” represents cells in CM only and “vehicle only”, cells in CM with 0.1% v/v of chloroform.

Concentration	$\beta$ -carotene	Astaxanthin
No treatment	0.2009 $\pm$ 0.0082	0.6436 $\pm$ 0.0065
Vehicle only	0.1993 $\pm$ 0.0050	0.6419 $\pm$ 0.0065
3 $\mu$ M	0.1968 $\pm$ 0.0066	0.6435 $\pm$ 0.0082
6 $\mu$ M	0.2067 $\pm$ 0.0067	0.6387 $\pm$ 0.0052
9 $\mu$ M	0.2082 $\pm$ 0.0093	0.6413 $\pm$ 0.0116
12 $\mu$ M	0.2058 $\pm$ 0.0049	0.6432 $\pm$ 0.0030

There was no significant difference between all treatments (Table 2.8):  $\beta$ -carotene (One-way ANOVA  $F = 1.66$ ,  $P > 0.05$ ,  $DF = 5$ ,  $N = 4$ ) and astaxanthin (One-way ANOVA  $F = 0.25$ ,  $P > 0.05$ ,  $DF = 5$ ,  $N = 4$ ).

## 2.4 DISCUSSION AND CONCLUSION

The objective of this chapter was to evaluate the tolerance levels of U937 cells to the various agents that will be used later on in this study for several purposes, using a measure of the cell membrane integrity. U937 cell biology is used in this study to investigate the aetiology and pathogenesis of schizophrenia and to explore the options for its treatment. A good recovery of cells at high viability from liquid nitrogen with a steady growth rate was obtained and established prior to all viability analysis. The Trypan Blue dye-exclusion and lactate dehydrogenase assays are both well-established methods of cell viability measurements. My choice of the LDH over the Trypan Blue method was because it limits human errors amongst other reasons.

Cytosolic lactate dehydrogenase release into the culture medium is a permeability assay differing from other methods because it is a non-invasive means of identifying membrane damage (Wroblewski and La Due, 1955). LDH activity is measured in cell and tissue supernatants by following the conversion of exogenously added lactate to pyruvate and simultaneously measuring the increase in absorbance resulting from the conversion of added NAD to NADH (Wroblewski and La Due, 1955). The assay is based on the premise that cellular damage will inevitably result in the loss of the ability to maintain and provide energy for metabolic function and growth. There was a significant correlation between Trypan Blue and LDH activity methods of cell viability measurements ( $R^2 = 0.94$ ,  $P < 0.05$ ) in cells exposed to 0 to 1 mM levels of hydrogen peroxide. The distinct advantage of the LDH assay is that numerous samples can be processed simultaneously and analyzed automatically using a microplate reader in reasonable short periods of time.

#### **2.4.1 Treatment with oxidants**

When confronted with stressors, chemical or environmental, organisms at both cellular and molecular levels respond to acclimatize themselves to their new environment. Oxidative stress is one such stimulus that can induce cellular adaptation. Lee and Um (1999), reported that the treatment of U937 cells with micromolar levels of  $H_2O_2$  for 8 – 24 h promotes resistance to a subsequent challenge with high concentrations that would otherwise be lethal. Hydroxyl radicals, formed from hydrogen peroxide, react very quickly with almost every type of molecule found in living cells: sugars, amino acids, phospholipids, DNA bases and organic acids. It is the most reactive oxygen radical known. During the ‘respiratory burst’ of phagocytes and many other cell types, some  $H_2O_2$  is released into the surrounding fluids. Provided that  $H_2O_2$  can be stopped from forming destructive  $OH^\bullet$  radicals, it may act as a useful intercellular signal between

cells. Low levels of oxidative stress, which can be achieved by the addition of  $\text{H}_2\text{O}_2$ , have been reported to stimulate *in vitro* cell proliferation. On the contrary, higher levels of oxidative stress usually decrease cell proliferation in culture and may have toxic effects.

$\text{H}_2\text{O}_2$  has been reported to be toxic to most cell types *in vitro* at or above 10 – 100  $\mu\text{M}$  levels (Halliwell and Gutteridge, 1999). This study however shows no significant effect on cell viability measured by both the LDH and Trypan Blue assays on exposure of U937 cells to 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h as reported by Lee and Um, 1999. It has been demonstrated that  $\text{H}_2\text{O}_2$  can elevate GSHPx levels in U937 cells, thus enhancing the cell's capacity to consume  $\text{H}_2\text{O}_2$  (Lee and Um, 1999).

It is known that macrophages including monocytes, can be activated to kill micro-organisms and tumour cells as an essential facet of immunity to intracellular parasites and tumours (Murray et al., 1983; Nathan et al., 1983; Bryne et al., 1986). Enhanced antimicrobial and antitumour activity has been shown to be dependent on an increased capacity of activated macrophages to produce toxic forms of oxygen, particularly  $\text{H}_2\text{O}_2$  (Adams and Nathan 1983; Murray, 1983). Macrophages produce toxic forms of oxygen by the activity of an NADPH oxidase similar to that of neutrophils. The oxidase contains FAD protein and a low-potential cytochrome  $\text{b}_{245}$  (Cross et al., 1983; 1985; Berton et al., 1985).

Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and hydroxyl radical, have been widely accepted as common signal mediators in a number of activation pathways (Finkel, 1998). An increased state of intracellular ROS is referred to as oxidative stress. Monocytes, as circulating blood cells, are easily affected by

dietary-derived peroxides and antioxidants present in the plasma. Devaraj et al., 1996, reported that the supplementation of diets with  $\alpha$  tocopherol in humans affects cellular function of monocytes. Moreover, monocyte cells, due to their involvement in inflammatory processes, are likely to find themselves in an oxidative environment *in vivo*. This study shows that the oxidant mixture of t-BHP and  $\text{FeSO}_4$  increases the loss of cell membrane integrity in U937 cells in a dose – and time – dependent fashion (Fig. 2.5). In a previous study, t-BHP on its own has been reported to kill U937 cells in a concentration (0 – 1.0 mM) and time dependent (after 6 and 24 h) manner (Guidarelli, 1996).

The exposure of hydroperoxides to transition metals such as  $\text{Fe}^{2+}$  results in the formation of further reactive radicals, including alkoxyl ( $\text{LO}\bullet$ ) and peroxy species (Gebicki and Gebicki, 1993; Davies et al., 1995), thus propagating peroxidative reactions. It is perhaps pertinent to note that U937 cells, as phagocytes are capable of generating oxidants for anti-microbial defence, and might therefore be expected to have enhanced antioxidant protection. As such, it is possible that the deleterious effect observed at 750: 750  $\mu\text{M}$  t-BHP:  $\text{FeSO}_4$  concentration on U937 cell membrane integrity was due to induced peroxidative damage.

#### **2.4.2 Treatment with organic solvents**

The careful selection of a solvent is a necessary requirement for the testing of water – insoluble compounds in a cell culture system. Testing of solvent toxicity must be evaluated prior to experiments (Crawford and Braunwald 1991). The results obtained in this study show that a concentration of 0.6% (v/v) of methanol and hexane; 0.8% (v/v) of ethanol has a toxic effect on cells. However, chloroform  $\leq 1\%$  (v/v) had no significant effect on cell membrane integrity.

Taken together, this study shows that organic solvents can be toxic to cells even at concentrations below 1% (v/v). Thus, ethanol was chosen as the solute for fatty acids at final concentrations  $\leq 0.6\%$  (v/v) and chloroform, though not toxic  $\leq 1\%$  (v/v) for carotenoids in cell suspensions.

#### **2.4.3 Treatment with fatty acids**

Fatty acids exist in every cell in the body in high quantities interacting with various cellular components, mainly the membrane. Changes in the relative amounts of free fatty acids in a membrane may contribute a major factor to the physiological role of the membrane. For example, in neuronal cells, the conduction of electrical signals along the axon is dependent on the exchange of ions between the inner and outer walls of the membrane. This process is largely dependent on the membrane fluidity, flexibility and permeability which in turn, control the physiological role of the ionic channel. Changes therefore in fatty acid levels ultimately affect membrane functions as shown in this study (Fig. 2.10). Since the presence of fatty acids above physiological levels is known to be cytotoxic, it is essential to use positive methods of addition of these substances as well as to determine their toxic levels in the system of interest. The concentrations of fatty acids used in this study may be found in the human plasma by dietary supplementation (Liebich et al., 1991). Fatty acids were also complexed to BSA and thus, these experiments were performed under similar physiological conditions. It has been suggested that PUFAs affect the proliferation of leukaemia cells *in vivo* as demonstrated in mice bearing the myeloid leukaemia T27A (Jenski et al., 1995). Since the U937 model is a leukaemia cell line, the loss of membrane integrity obtained with 40  $\mu\text{M}$  PUFAs over 3 days could result from this. However, a better biochemical understanding of how PUFAs affect cell membrane integrity and ultimately cell death is necessary. Although several studies have suggested a protective role of fatty acid

binding proteins against high intracellular FA concentrations (Kaikaus et al., 1990; Veerkamp et al., 1991; Vork et al., 1993; Glatz and van der Vusse, 1996), or even free radicals (Samanta et al., 1989), this and the study by Zimmerman and Veerkamp (2001), show no evidence for such roles.

In a study by Finstad et al. (1998), it was found that PUFAs especially AA, EPA and DHA reduced cell multiplication, loss of membrane integrity and caused DNA fragmentation in some leukaemia cell lines including U937-1 cells when used above certain levels. However, these responses were reported to be dependent on cell type.

This study demonstrates no significant difference in LDH leakage in U937 cells incubated for 24 h with 0 to 40  $\mu\text{M}$  of SA, OA, AA, EPA and DHA compared to controls when complexed with fatty-acid-free BSA in the molar ratio 2.5 : 1 (Tables 2.3 and 2.4). After the treatment with 10  $\mu\text{M}$  of these five fatty acids for 72 h, there was no significant increase in the loss of membrane integrity. However, when these cells were incubated with 40  $\mu\text{M}$  for 72 h, there was a significant difference between treatments (Fig. 2.10). There was no difference in LDH parameter obtained with 40  $\mu\text{M}$  SA or OA compared to controls. A striking effect however, was that the greatest damage was obtained with cells incubated with 40  $\mu\text{M}$  EPA and not 40  $\mu\text{M}$  DHA after 3 days. Finstad et al., (1998), reported negligible number of cells with leaky membranes after 3 days when treated with 60  $\mu\text{M}$  EPA but appreciable at concentrations  $\geq 120$   $\mu\text{M}$ . In contrast, the present study shows a significant increase in LDH leakage in U937 cells when treated with 40  $\mu\text{M}$  EPA for 3 days. A possible explanation for this is that the EPA used was partially oxidised as packaged by the manufacturer. However, this possibility appears not to be the case as shown in subsequent chapters. This study shows that the treatment of U937 cells with 40  $\mu\text{M}$  PUFAs (AA, EPA and DHA) for 3

days increased the loss of membrane integrity significantly while SA and OA had no significant effect at that same concentration.

To investigate if the increase in LDH activity in cells incubated with 40  $\mu$ M PUFAs were reversible upon removal of these fatty acids from the medium, cells were incubated for 3 days with PUFAs (AA, EPA or DHA). The cells were then washed, diluted to a density of  $0.5 \times 10^6$  cells/ ml and re-incubated in a fatty-acid-free medium for three more days. After pre-incubation for three days LDH leakage was higher in cultures exposed to AA, EPA and DHA but not with SA or OA compared to controls (Fig. 2.10). Re-incubation of cells in a fatty-acid-free medium for three more days showed reduced toxicity of the PUFAs (AA, EPA and DHA), with the U937 cells on the road to recovery. The prolonged presence or withdrawal of SA or OA had no effect on LDH leakage. Similarly, Finstad et al. (1998), reported a reversible effect of EPA when U937 cells were re-incubated in an EPA-free medium.

Finstad et al., (1998), found a reduction in cell number and an increased degree of apoptosis in cells treated with 60  $\mu$ M EPA after 3 days and these effects were not counteracted by pre-treating these cells with antioxidants such as vitamin E and C, suggesting that the damage was not oxidatively induced. It is however possible that PUFAs may interfere with cell proliferation of cancer cells by the formation of lipid peroxidation by-products (Chajes et al., 1995). This study has demonstrated that polyunsaturated fatty acids can reduce membrane integrity of U937 cells at certain concentrations over prolonged periods. The extent of cell membrane damage has been reported to vary from one cell line to another (Finstad et al., 1998).

Even though re-incubation of cells in a fatty-acid-free medium for three more days did not completely reverse the damaging effects of PUFAs on cell viability, there was a significant decrease in LDH leakage compared to the results obtained in the presence of PUFAs. This suggests that prolonged withdrawal of these FAs may eventually lead to a complete reversal producing similar results obtained to that of controls. For follow-up experiments, U937 cells were incubated with 0 – 40  $\mu$ M fatty acids concentrations for times  $\leq$  24 h, unless otherwise stated and with 10  $\mu$ M levels for time periods over 24 h.

#### **2.4.4 Treatment with antioxidants**

##### **(A) *With ascorbic acid (Vitamin C)***

It is well established that mammalian cells lack the ability to synthesize ascorbic acid but express transport systems that mediate the cellular uptake of the vitamin (Guidarelli, 2001). In solution ascorbic acid undergoes metal-catalysed oxidation to dehydroascorbic acid, with the concomitant formation of superoxides and hydrogen peroxide (Carr and Frei, 1999). Ascorbic acid (Vitamin C) is a major water soluble antioxidant, found in the aqueous compartments of cells and extracellular fluids, capable of scavenging reactive oxygen species (Chou and Khan, 1983; Chesney et al., 1991; Buettner and Jurkiewicz, 1996).

Vitamin C, also known as ascorbic acid, occurs at concentrations between 30 and 100  $\mu$ M levels in the human plasma. Millimolar levels in different cell types are sufficient to exert antioxidant effects (Halliwell and Gutteridge, 1999). This study shows that no significant increase in LDH activity at 0 – 500  $\mu$ M ascorbic acid levels. However, concentrations of ascorbic acid that will subsequently be used will vary from 0 – 400  $\mu$ M because there was no significant increase in LDH leakage in U937 cells at these levels.



### **(B) *α-tocopherol***

α-tocopherol, a potent fat-soluble antioxidant vitamin sequestered in the hydrophobic interior of membranes, quenches lipid peroxidation (Burton et al., 1983). It has been suggested that the distribution of vitamin E reflects the degree of its saturation in membrane lipids (Buttris and Diplock, 1988). However, there is a controversy as to the exact subcellular distribution of vitamin E. Mitochondria are generally found to be relatively rich in vitamin E, but its distribution between the inner and outer membranes is still unclear (Oliveira et al., 1969; Buttris and Diplock 1988). There was no significant difference in LDH leakage in cells incubated with 0 – 50 μM levels of vitamin E for 24 h. However concentrations < 50 μM will be used henceforth in this study.

### **(C) *With Carotenoids***

Carotenoids are water insoluble antioxidants found in nano-molar concentrations in human serum and breast milk (Khachik et al., 1997). For this study a member of the two carotenoids groups was employed: β-carotene for hydrocarbons and astaxanthin for xanthopylls. Concentrations of β-carotene and astaxanthin varying from 0 to 12 μM showed no significant changes in cell membrane integrity after 24 h treatment. Carotenoids are unstable and therefore stock solutions change over time due to oxidative breakdown (Hess et al., 1991). Being highly hydrophobic, carotenoids show a strong tendency to aggregate and crystallise in aqueous media. The accumulation of carotenoids as micro - crystalline aggregations is common in the chloroplasts of higher plants (e.g. lycopene in tomato) (Pfander et al., 1994). Aggregation changes the physical properties of carotenoids (light absorption and chemical reactivity). Changes in carotenoid size and ease of solubilization affect the ease of absorption and bio-availability in animals and their ability to enter and function in sub-cellular structures

(Gruszecki, and Sielewiesiuk, 1990). In this study, the carotenoids used were dissolved in chloroform and sonicated briefly to improve the ease of absorption into cellular fractions whilst preserving its stability. Carotenoid concentrations, therefore, used subsequently in following chapters of this study ranged from 0 to 12  $\mu$ M because these levels did not affect cell membrane integrity.

#### **2.4.5 Conclusion**

In conclusion, this chapter demonstrates that U937 cells are fast dividing cells with a steady growth when maintained at 0.5 to 1.0 x 10<sup>6</sup> cells/ml. It also shows a good correlation between the lactate dehydrogenase activity quantified in culture medium and the Trypan Blue dye exclusion method of cell viability measurements. In addition, it shows the sensitivity of U937 cells to PUFAs especially EPA. Therefore, to evaluate the possible use of n-3 fatty acids in future schizophrenia therapy, a better understanding of the biochemical mechanisms responsible for the loss of membrane integrity is required.

## CHAPTER 3: FATTY ACID INCORPORATION INTO CELLULAR

### PHOSPHOLIPIDS

#### 3.1 INTRODUCTION

Fatty acids are essential components of cell membrane phospholipids. They determine membrane properties and modify signal transduction mechanisms across cell membranes. These include pathways involving the formation of second messengers after the enrichment of cells with n-3 and n-6 polyunsaturated fatty acids (PUFAs) (Swann et al., 1990; Weber et al., 1991). Free fatty acids (FFAs) are rapidly and completely absorbed into membrane lipid fractions with large chain fatty acids, normally more than 99%, bound to serum proteins (Fenton et al., 2000). In the brain, the binding of FFAs to proteins is accompanied by a swift dissociation rate and exchange of unbound and unincorporated essential fatty acids between the brain and blood. As such, the uptake of FFAs by the brain is buffered against short-term fluctuations in blood levels, primarily reflecting the metabolic needs of the brain for these fatty acids (Banks et al., 1997).

Of the polyunsaturated fatty acids, docosahexaenoic acid (22: 6n-3) is the longest and most unsaturated fatty acid commonly found in the phospholipids of biological membranes. It represents a major component of membranes of a few specialized tissues including the brain, sperm and retinal rod outer segments, where DHA can account for up to 50 mol% of the total fatty acids (Hendriks et al., 1976; Salem et al., 1986).

The goal of this qualitative study was to determine whether the different fatty acid supplements were incorporated into the phospholipids of U937 cells after the enrichment of the culture medium with 40  $\mu$ M levels of SA, OA, AA, EPA or DHA and incubation for 24 h.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Materials**

Fatty-acid-free bovine serum albumin (BSA), stearic acid (SA), oleic acid (OA), arachidonic acid (AA), phosphatidyl choline, 0.4% Trypan Blue, Hanks balanced salt solution (HBSS), RPMI 1640, foetal bovine serum (FBS), 200 mM L-glutamine, 50 mg/ml gentamicin solution, HPLC-grade chloroform, hexane, diethyl ether, methanol and ethanol, glacial acetic acid, potassium chloride (KCl), 2', 7'-dichlorofluorescein, sulfuric acid, toluene, potassium hydrogen carbonate (KHCO<sub>3</sub>), butylated hydroxytoluene (BHT) and nonadecanoic acid methyl ester were purchased from Sigma-Aldrich, UK. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were from Nu-chek Prep., Denmark. LK5D – Whatman TLC plates were supplied by Fisher Scientific, UK.

### **3.2.2 U937 cell culture**

U937 cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine and 0.05 mg/ml gentamicin at 37°C in a humidified incubator with 5% CO<sub>2</sub>/95% air. For all experiments, cells were maintained at  $0.5 \times 10^6$  cells/ml at viability > 90% by Trypan Blue dye exclusion.

### **3.2.3 Treatment of cells with fatty acids**

$1.5 \times 10^7$  cells at  $0.5 \times 10^6$  cells/ml were supplemented with 40  $\mu$ M (final concentration) of SA, OA, AA, EPA or DHA using aliquots of 10 mM FA stock solutions. FAs were complexed with BSA at a molar ratio of 2.5:1. Control cells were incubated with ethanol/BSA complex. Cells were incubated in 30 ml suspensions in 75 cm<sup>2</sup> culture flasks for 12 h to allow for the incorporation of fatty acids into cellular phospholipids. Thereafter, a homogenous cell mixture was ensured by careful aspiration and dispensing

using a Pasteur pipette. 10 ml of cell suspension was transferred into 15 ml centrifuge tubes and FA uptake was terminated by centrifugation at 4000 g for 5 min at 4°C. Cells were washed twice each time with 10 ml of HBSS and re-suspended in 0.2 ml of ice-cold HBSS.

### **3.2.4 Lipid extraction**

Extraction of total cellular lipids from pelleted cells was performed by a modified method of Folch et al. (1957). Lipids in 0.2 ml of cell pellets were extracted with 0.8 ml of ice-cold chloroform (C): methanol (M) (2: 1 v/v) in 1.5 ml eppendorf tubes. The cell suspension was sonicated for 3 x 10 s with 10 s intervals in an ice-bath followed by the rapid addition of 0.2 volumes of ice-cold methanol (0.2 ml) to reduce the density of the extracting solvent (Radin, 1988). Suspensions were mixed briefly with a vortex mixer and centrifuged at 4000 g for 3 min at 4°C. Centrifugation was used instead of filtration to convert all non-lipid residues into compact pellets due to the small sample size. An aliquot of 0.8 ml of the supernatant, containing the lipid residue, was transferred into labelled, sterile Pyrex tubes and lipid extraction was repeated as previously performed but this time, 1.05 ml of supernatant was carefully drawn. The two supernatants were combined and the pellets were discarded.

Thereafter, 0.88% KCl was added as 25% of the existing volume (0.5 ml). Tubes were shaken and allowed a few minutes for separation into two phases. 0.8 ml of the organic (bottom) layer was transferred into a clean vial and lipids left in the Pyrex tubes were re-extracted by the addition of 800 µl of C: M (2: 1, v/v with 0.01% BHT). 0.6 ml of the bottom layer was drawn, combined with the previous extract and evaporated to dryness under a gentle stream of nitrogen (N<sub>2</sub>). The extract was re-suspended in 0.03 ml of C: M (2: 1, v/v) with 0.01% BHT and stored at -20°C.

### 3.2.5 Thin layer chromatographic separations

A solvent system of hexane: diethyl ether: acetic acid, 70: 30: 1.3 (v/v/v) was prepared. Using a micro-Pasteur pipette, 20  $\mu$ l aliquots of lipid extracts were spotted on LK5D plates under a stream of N<sub>2</sub> with reference phosphatidyl choline standard on a separate lane for identification purposes. The plates were run in a chamber pre-saturated with 100 ml of the solvent system above. After the solvent front reached 1–2 cm from the top, plates were dried under a stream of N<sub>2</sub> until free of acetic acid odour. Afterwards, plates were sprayed with 0.1% (w/v) 2', 7'-dichlorofluorescein in ethanol. Lipid spots corresponding to standard phospholipid bands were viewed and marked under UV lights and scraped into clean Pyrex tubes.

### 3.2.6 Conversion of lipids into methyl esters (FAMES)

To each tube (above), 2 ml of 1% sulfuric acid in methanol (v/v) and 1 ml of toluene were added to solubilize the triacylglycerols. Each tube was flushed with N<sub>2</sub>, mixed by vortexing in tubes tightly sealed with Teflon-lined screw caps and heated at 50°C overnight. After chilling the tubes briefly on ice, 5 ml of hexane containing 0.01% BHT and 2 ml of KHCO<sub>3</sub> (2%, w/v) were added. After thorough shaking of tubes, 4 ml of the upper organic layer was transferred into pre-weighed vials and evaporated to dryness. Lipid extraction was repeated by the addition of 3 ml of hexane containing 0.01% BHT and 1 ml of KHCO<sub>3</sub> (2%, w/v). 3 ml of the upper layer was combined with the previous extract and re-evaporated to dryness under nitrogen. Vials were re-weighed and the total FAMES weight was calculated as the difference between the two weights. Methyl ester extracts were stored in hexane at a concentration of 10 mg/ml with nonadecanoic acid methyl ester (C19: 0) used as the internal standard, added as 10% of the total mass of FAMES in each sample (using 1  $\mu$ M stock solution in hexane).

Fatty acid peaks were identified according to the retention times of standard compounds of methyl esters and quantified using C19: 0 as internal standard.

### **3.2.7 Gas-liquid chromatography**

Fatty acid methyl esters were analysed on a Perkin-Elmer 8320 gas chromatograph fitted with a capillary column (Zebron ZB-WAX, 100% polyethylene glycol, 30 m, 0.25 mm ID, 0.25  $\mu$ m FD and hydrogen as the carrier gas at 33 cm/s). The oven temperature was programmed from 160 °C to 240 °C at 4°C/ minute, holding at 240 °C for 10 minutes. The flame ionization detector (FID) temperature was set to 300 °C, an injection volume of 2  $\mu$ l, injection temperature of 290 °C and a split ratio of 100:1. Fatty acids were identified according to the retention times of standard fatty acid methyl esters and quantified using C19:0 as the internal standard.

## **3.3 RESULTS AND DISCUSSION**

The phospholipid fatty acid profile demonstrates that untreated U937 cells are relatively rich in the saturated - (14:0, 16:0 and 18:0) and monounsaturated - (16:1n-7; 18:1n-7 and 18:1n-9) fatty acids whereas, they contain low levels of the polyunsaturated fatty acids (20:4, 20:5 and 22:6) (Table 3.1). The supplementation of culture medium with 40  $\mu$ M SA, OA, AA, EPA or DHA led to a rapid increase in the levels of the respective fatty acids incorporated into the phospholipid fractions of U937 cells. Yano et al., 2000 showed that elevation in PUFA concentrations in cellular phospholipids was accompanied by decreases in the levels of the saturated- and monounsaturated fatty acids found in U937 cell phospholipids.

The incorporation of fatty acids especially the PUFAs (AA, EPA and DHA) into phospholipid fractions of U937 cells has previously been studied in detail (North et al.,

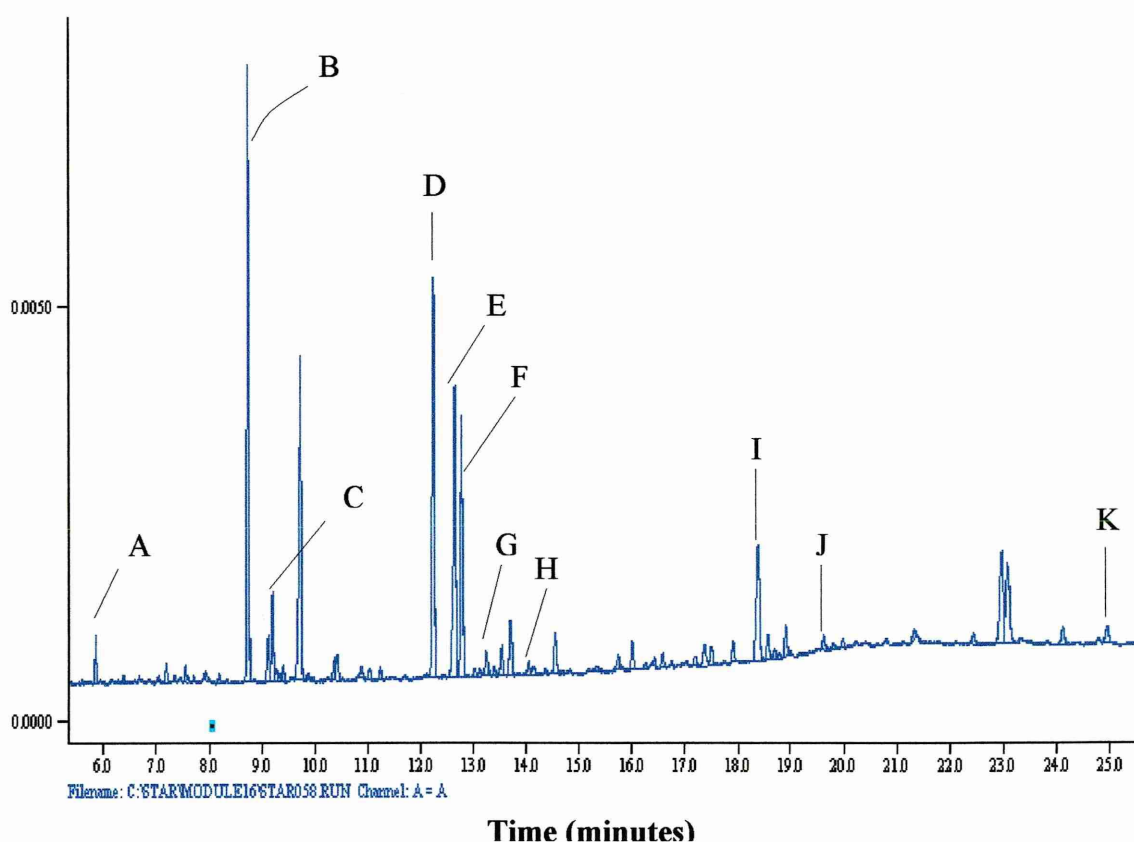
1994; Obermeier et al., 1995; Yano et al., 2000). As such, this study was limited to demonstrating that these fatty acids (SA, OA, AA, EPA and DHA) are indeed readily and rapidly taken up by U937 cells in culture. Although the present study suggests that AA or EPA incorporated into cellular phospholipids post supplementation was higher than DHA levels, it is important to bear in mind that this study was not replicated. More so, previous studies have shown that DHA is easily taken up into U937 cell phospholipids (North et al., 1994; Obermeier et al., 1995; Yano et al., 2000).

**Table 3.1** Fatty acid composition of the phospholipid fraction of U937 cells supplemented with or without 40  $\mu$ M SA, OA, AA, EPA, DHA or ethanol (controls) for 12 h. Values were expressed in  $\mu$ M based on the concentration of internal standard added. Values represent data from a single experiment (n = 1).

FA ( $\mu$ M)	Fatty acid supplement					
	Control (None)	SA 18: 0	OA 18: 1 (n-9)	AA 20: 4 (n-6)	EPA 20: 5 (n-3)	DHA 22: 6 (n-3)
14:0	0.87	0.41	0.33	0.56	0.51	0.42
16:0	14.36	9.47	8.96	5.29	5.98	6.00
16:1 (n-7)	2.32	0.75	0.82	1.07	1.02	0.85
18:0	3.79	<b>9.20</b>	5.06	6.62	5.19	4.22
18:1 (n-7)	2.41	2.08	2.04	3.26	4.81	2.65
18:1 (n-9)	3.09	3.49	<b>8.36</b>	3.80	3.69	2.62
18:2 (n-6)	0.27	0.20	0.20	0.23	0.19	0.30
20:4 (n-6)	1.55	1.28	1.10	<b>4.36</b>	1.96	1.34
20:5 (n-3)	0.20	0.39	0.20	0.26	<b>4.41</b>	0.59
22:6 (n-3)	0.23	0.17	0.20	0.28	0.18	<b>1.50</b>



Since U937 cells normally have low levels of the polyunsaturated fatty acids (Fig. 3.1) namely: AA, EPA and DHA (North et al., 1994; Obermeier et al., 1995; Yano et al., 2000), supplementation of the culture medium with these PUFAs is required to increase the PUFA contents of these cells.



**Fig. 3.1** Gas chromatographic traces of fatty acid methyl esters of control U937 cell phospholipids in culture medium without fatty acid supplementation. A: myristic acid (14:0); B: palmitic acid (16:0); C: palmitoleic acid (16:1n-7); D: stearic acid (18:0); E: oleic acid (18:1n-9); F: vaccenic acid (18:1n-7); G: linoleic acid (18:2); H: internal standard (19:0); I: arachidonic acid (20:4n-6); J: eicosapentaenoic acid (20:5n-3); K: docosahexaenoic acid (20:6n-3).

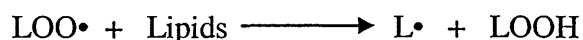
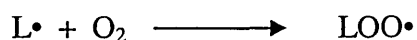
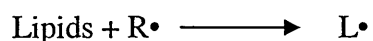
### **3.4 Conclusion**

In conclusion, this study has shown that U937 cells are capable of incorporating freely available fatty acids including the PUFAs (AA, EPA and DHA) into their cellular phospholipids after 24 h of incubation at 37°C and 5% CO<sub>2</sub> in a humid environment. This information shows that these cells possess basal phospholipase and lysophospholipase activity under normal incubation conditions.

## CHAPTER 4: LIPID PEROXIDATION EXPERIMENTS (MEASUREMENTS OF LIPID HYDROPEROXIDES)

### 4.1 INTRODUCTION

Lipids have fundamental structural and functional roles in all membranes including those of neuronal cells. Lipid peroxidation in biological membranes is considered to be one of the major mechanisms of cellular injury in organisms subjected to oxidative stress (De Zwart et al., 1999). The oxidation of PUFA-rich lipids and their susceptibility to oxidative damage varies between humans, their red blood cells, low-density lipoproteins, in health and disease states. Lipid peroxidative stress in the nucleus may contribute to histone modification such as amino acid oxidation, protein-protein cross links and DNA-protein cross links. Nucleosome histones protect the DNA from free-radical mediated damage (Enright et al., 1992) and histone detachment/re-attachment are closely linked with transcription and replication. Furthermore, this “shuttling of histones” contributes to DNA repair and thus requires functionally intact proteins. It has been demonstrated that oxidatively-modified histones have the ability to damage DNA directly by cross linkage (Jones et al., 1993; Altman et al., 1995). Therefore, the selective and efficient degradation of oxidatively damaged nuclear proteins, possibly due to lipid peroxidation, appears to be essential for the maintenance of genomic integrity (Ullrich et al., 2000). The chemical mechanism of lipid peroxidation involves three stages: initiation, propagation and termination.

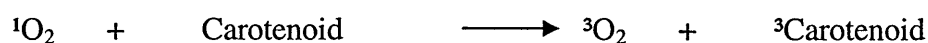


Where  $\text{R}\cdot$  is the radical initiator,  $\text{L}\cdot$ , the lipid radical,  $\text{LOO}\cdot$ , the lipid peroxide radical and  $\text{LOOH}$ , the lipid hydroperoxide.

It is now widely accepted that cellular damage induced by uncontrolled iron-catalysed free-radical-mediated oxidative stress is implicated in an array of pathological diseases including inflammatory disorders (Blake et al., 1983; Trenam et al., 1992), atherosclerosis (Smith et al., 1992; Witztum, 1994) and neurodegenerative disorders (Olanow and Arendash, 1994; Behl et al., 1994; Gerlach et al., 1994; Gassen and Youdim, 1997). The generation of free radicals and subsequent oxidative modification of biomolecules such as lipids, proteins and nucleic acids is inevitable for aerobic organisms. While low levels of free radicals have proved to be involved in important physiological functions such as signal transduction leading to gene expression and cell proliferation (Suh, et al., 1999; Brar, et al., 1999), excessive amounts have been implicated in a variety of pathological conditions including schizophrenia (Mahadik and Mukherjee, 1996a; Ramchand et al., 1996; Mahadik et al., 2001). Schizophrenia is a serious mental disease, the aetiology of which is yet unknown. Growing evidence indicates that disturbed phospholipid metabolism contributes to the disorder (Horrobin, 1996). Several studies have reported controversial results in the levels of essential fatty acids in schizophrenic patients but more consistent is the finding of reduced linoleic acid (18: 2 n-6), AA (20: 4n-6) and DHA (22: 6n-3) in erythrocyte membranes and plasma of schizophrenic patients (Glen et al., 1994; Peet et al., 1995; Fenton et al., 2000). Since the brain has high oxygen consumption, and is rich in lipids and transition metals, it is at particular risk of oxidative damage. Elaborate antioxidant defense systems exist therein to protect against oxidative stress. Lipid peroxidation has also been implicated in schizophrenia by the finding of increased levels of TBARS and exhaled pentane in expired air (Kovaleva et al., 1989; Phillips et al., 1993), suggesting an increase in membrane phospholipid breakdown (Glen et al., 1994).

Free radicals are unstable molecules implicated in many neurodegenerative diseases such as schizophrenia, Alzheimer's disease and Parkinson's disease. Just as free radical damage to heart and blood vessels may lead to heart attacks (Heinle et al., 2002), attack on brain cells may impair mental acuity over time. In fact, the brain may bear the brunt of free radical damage since it is highly enriched with PUFAs, a favourite target for these radicals.

Biological systems have evolved complex protective strategies against free radical-induced damage, which is kept in check by the antioxidant defense system, comprising the enzymatic and non-enzymatic components. The putative nature of this system involving antioxidant dietary nutrients such as ascorbic acid,  $\alpha$ -tocopherol and the carotenoids (such as  $\beta$ -carotene and astaxanthin), could disarm the harmful free radicals before the damage occurs in neuronal cells.  $\beta$ -carotene (for the carotenes) and astaxanthin (for the xanthophylls) were used as medium supplements in this study to determine the antioxidant activities of carotenoids. Burton and Ingold, (1984), first proposed the mechanism by which carotenoids might quench lipid radicals in biological cell membranes. It involves the transfer of excitation energy from singlet oxygen ( $^1\text{O}_2$ ) to the carotenoid, resulting in the formation of the carotenoid triplet (Krinsky, 1988):



In a subsequent reaction, the excitation energy is harmlessly dissipated through rotational and vibrational interactions between the carotenoid triplet and the solvent, thus regenerating the original carotenoid molecule:



In this way, carotenoids can act in a catalytic manner, neutralizing the potentially harmful singlet oxygen molecule (Foote et al., 1970), and thereby function as an antioxidant preventing oxidation reactions. The limits on this type of protection appear to be the number of conjugated double bonds in the molecule, with a maximum protection being seen with those having nine or more such bonds. The presence of additional functional groups on these molecules, such as allylic or non-allylic hydroxyl groups, does not seem to alter the quenching characteristics of carotenoid pigments (Krinsky, 1995).

If carotenoids functioned truly in a catalytic fashion in this reaction, cells and animals could obtain a lifetime supply of protection by synthesizing or ingesting a sufficient supply of carotenoid pigment to quench all of the available  $^1\text{O}_2$ . However, this is not possible because there is a chemical reaction in addition to the physical reaction which limits the ability of carotenoids to protect against  $^1\text{O}_2$  (Foote 1970) thus destroying the carotenoid molecule in the process. Under physiological oxygen tensions, the reaction rate of carotenoids with peroxyl radicals appears to be sufficient to prevent the abstraction of allylic hydrogens from neighboring lipids thus, inhibiting lipid peroxidation (Burton, 1989).

U937 cells are known to contain glutathione (GSH), a non-enzymatic antioxidant (Nardini et al., 1998). T-BHP-induced oxidative stress has been reported to result in the depletion and oxidation of intracellular GSH in U937 cells (Latour et al., 1995; Nardini et al., 1998). It is generally accepted that ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin act as antioxidants in the protection of biological membranes from free radical-mediated damage. Ascorbic acid (vitamin C) concentration in healthy human plasma is about 200  $\mu\text{M}$  with the brain having the highest concentration compared to

other tissues (Mahadik and Scheffer, 1996).  $\alpha$ -tocopherol, like the carotenoids  $\beta$ -carotene and astaxanthin are lipid-soluble and therefore effectively prevent plasma and lipid peroxidation (Burton and Ingold, 1984; Terao, 1989; Burton and Ingold, 1990).

Although the detailed mechanisms by which free radicals lead to cellular oxidative damage are not yet fully understood, lipid peroxidation in cell membranes is considered to be critically involved (De Zwart et al., 1999). There are several ways of monitoring lipid peroxidation, including: the determination of oxygen uptake, loss of lipid substrates such as PUFAs and the accumulation of peroxidation by-products such as hydroperoxides and thiobarbituric acid reactive substances (TBARS). As shown in chapter three, U937 cells are relatively poor in polyunsaturated fatty acids. Thus, culture medium was supplemented with PUFAs with or without antioxidants before the induction of oxidative stress. In this chapter, the susceptibility of biological samples to lipid peroxidation was determined by the exposure of PUFA-enriched U937 cells to the lipid peroxidation initiator (t-BHP/ $\text{Fe}^{2+}$ ) and lipid hydroperoxides produced were measured colorimetrically. The antioxidant efficiency of ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin in preventing the accumulation of lipid hydroperoxides in PUFA-enriched U937 cells was also studied.

Tissue measurements of lipid peroxidation in systems exposed to oxidative stress differ considerably in the sensitivity of the method employed. It may be determined by measuring the initial products of lipid peroxidation such as conjugated dienes and lipid hydroperoxides or the lipid peroxidation breakdown products such as aldehydes. The method employed in this study detects lipid hydroperoxides using the ferrous oxidation/Xylenol orange (FOX) method (Jiang et al., 1991). Samples were subjected to oxidative stress by a system containing tert-butyl hydroperoxide (t-BHP) and  $\text{Fe}^{2+}$ .

Iron, though required for normal cell growth and proliferation, is potentially harmful in excessive amounts, catalysing the formation of free radicals via Fenton chemistry (Linn, 1998). The exposure of hydroperoxides to transition metal ions such as copper or iron yields further reactive radicals including alkoxyl ( $\text{LO}\cdot$ ) and peroxy ( $\text{LOO}\cdot$ ) radicals (Davies, 1996). Studies have shown that lipid hydroperoxides can give rise to further oxidative damage by propagative processes (Chapter one). U937 cell response to oxidative injury induced by t-BHP (an organic hydroperoxide) and  $\text{Fe}^{2+}$ , was investigated. Since it has previously been shown that U937 cells lose their cell membrane integrity in response to an oxidising mixture of t-BHP and  $\text{FeSO}_4$  (Chapter two), this chapter aims to examine the relationship between this loss and the accumulation of lipid hydroperoxides in cell membranes of models.

Xylenol orange is a good chelator and quantitative indicator of a wide range of cations, including iron, binding  $\text{Fe}^{3+}$  but not  $\text{Fe}^{2+}$ , at acidic pH, giving a coloured Fe (III)-xylenol orange complex (Gay et al., 1999). This reaction is insensitive to oxygen concentrations. To date most methods available for the measurement of lipid peroxidation have limitations either of reproducibility, sensitivity, rapidity or accuracy. For instance, the popular TBARS assay has been criticised for its lack of specificity and accuracy; and the nonexclusive, indirect and inefficient nature of MDA formation from lipid oxidation products. Since MDA is also a secondary product of oxidative injury to DNA and perhaps, other non-lipid biomolecules, its formation cannot be automatically indicative of lipid peroxidation in complex systems containing any potential non-lipid MDA source. This is particularly true if the system is exposed to harsh oxidative conditions primarily for the derivatization of MDA (Janero, 1990).



The goal of the present study was to investigate the sensitivity to induced peroxidative damage, in U937 cells, with or without fatty acid modifications of their cellular phospholipids. In addition, the study examined the effects of some dietary antioxidants on the susceptibility of these cells to oxidative stress, *in vitro*. This study describes a modified version of the FOX assay (Jiang et al., 1991), a rapid, sensitive and cost-effective method for the measurement of lipid hydroperoxides (LOOH). The assay is based on the oxidation of  $\text{Fe}^{2+}$  by LOOH, in the presence of the dye, xylene orange.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Materials**

Xylene orange (*o*-cresolsulfonephthalein-3-3'-bis-(methyliminodiacetic acid sodium salt), ammonium iron (II) sulfate hexahydrate, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), tert-butyl hydroperoxide (t-BHP), iron sulfate ( $\text{FeSO}_4$ ), butylated hydroxytoluene (BHT), stearic acid (SA), oleic acid (OA), arachidonic acid (AA), ascorbic acid, vitamin E,  $\beta$ -carotene, astaxanthin, sulfuric acid ( $\text{H}_2\text{SO}_4$ ) and HPLC-grade methanol (99.9%) and ethanol (99.9%) were purchased from Sigma-Aldrich, UK. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were obtained from Nu-chek Prep., Denmark.

### **4.2.2 Glass and plastic preparations**

All glassware used was washed in de-con and nano-pure water, autoclaved at 121°C for 30 min and dried in an oven before use. All other apparatus used were sterile with all experiments (prior to absorbance measurements) performed under a laminar flow hood (class II).

### **4.2.3 Ferrous oxidation/xylene orange (FOX) assay**

#### **4.2.3.1 Preparation of FOX reagent**

1000 ml of reagent was prepared containing 900 ml of pure methanol, 100 ml of 0.25 M  $\text{H}_2\text{SO}_4$ , 0.88 g of BHT (in order to inhibit further peroxidation within the assay itself), 0.076 g of xylene orange, and 0.098 g of ammonium iron (II) sulfate hexahydrate. The FOX reagent was stored in Schott bottle at 2 – 8°C.

#### **4.2.3.2 FOX assay**

The standard assay protocol used for all U937 cell extracts follows: samples were sonicated for 3 x 10 s in an ice bath with an interval of 10 s and centrifuged at 250 g for 2 min at 4°C. Thereafter, a sample: reagent mixture was prepared in a 1: 9 (v/v) ratio by transferring 0.02 ml (20  $\mu\text{l}$ ) of the supernatant into each well of a 96-well plate and adding 0.18 ml (180  $\mu\text{l}$ ) of the FOX reagent using a multi-channel pipette. Plates were carefully sealed with pressure-sensitive Costar plate-sealers and lids, and then wrapped with aluminium foil paper and incubated in the dark at room temperature for 12 – 24 h (overnight) unless stated otherwise. Absorbance at 560 nm was read using the EL340 microplate reader and lipid peroxide concentrations calculated from Beer's law using an apparent extinction coefficient of  $4.3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  for t-BHP at 560 nm (Jiang et al., 1991).

### **4.2.4 Method validation**

#### **4.2.4.1 Maximal time points**

In order to determine the maximum incubation time at room temperature required for the formation of Fe (III)-xylene orange complex, standard peroxides were used.

**(A) With hydrogen peroxide ( $H_2O_2$ )**

A stock solution of 200 mM  $H_2O_2$  was prepared and further diluted to 2 mM and then 0.3 mM concentrations. All  $H_2O_2$  solutions were stored in dark bottles at 2 – 8°C. 20  $\mu$ l of 0.3 mM  $H_2O_2$  was transferred into each well of a 96 – well plate and mixed with 180  $\mu$ l of FOX reagent (1: 9, v/v). The absorbance at 560 nm was immediately read over 45 min at room temperature using the EL340 microplate reader. The final  $H_2O_2$  concentration per well measured over time was 30  $\mu$ M.

**(B) With t-BHP:  $FeSO_4$**

0.2 M t-BHP and 0.6 mM  $FeSO_4$  stock solutions were prepared in nano-pure water just before use. 0.2 M t-BHP was further diluted to 0.6 mM in water. 10  $\mu$ l each of 0.6 mM t-BHP and  $FeSO_4$  was mixed and transferred into a 96-well plate, and 180  $\mu$ l of the FOX reagent was added using a multi-channel pipette. In order to determine the maximum time required for the formation of Fe (III)-xylenol orange complex, absorbance was read at 560 nm at the indicated times over 24 h with the EL340 microplate reader at room temperature. Final concentration of t-BHP:  $FeSO_4$  per well was 30: 30  $\mu$ M.

**(C) With increasing  $FeSO_4$  levels**

A stock solution of 10 mM  $FeSO_4$  was prepared just before use. Concentrations varying from 0 to 1000  $\mu$ M were prepared by serial dilution to a final volume of 20  $\mu$ l, which was mixed with 180  $\mu$ l of the FOX reagent in 96-well plates. Plates were incubated at room temperature for 30 min in the dark before absorbance was read at 560 nm.

#### **4.2.5 Cell culture**

U937 cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine and 0.05 mg/ml gentamicin at 37°C in a humidified incubator with 5% CO<sub>2</sub>/95% air. For all experiments, cells were maintained at 0.5 x 10<sup>6</sup> cells/ml at viability > 90% by the Trypan Blue dye-exclusion method of viability measurements.

#### **4.2.6 Treatment of cells with t-BHP: FeSO<sub>4</sub>**

Stock solutions of 2 mM t-BHP and 5 mM FeSO<sub>4</sub> were prepared fresh just before use.

Oxidative stress was induced by the addition of 0.9 ml of freshly prepared oxidant mixture in HBSS (or HBSS only as the control) to 0.1 ml of HBSS - washed cell suspension in 24-well plates. Incubations followed at 37°C and 5% CO<sub>2</sub> in a humid environment. For the dose-dependent assay, cells were incubated with 0: 0 to 1000: 1000 µM final concentrations of t-BHP: FeSO<sub>4</sub> for 30 min under the conditions stated above. In order to determine the incubation time required for the generation of a detectable, significant increase in the amount of lipid hydroperoxides produced in U937 cells incubated at 37°C under oxidative stress, a time course experiment was carried out with a final oxidant concentration of 750: 750 µM t-BHP: FeSO<sub>4</sub> over 24 h. The 0 h experiment involved an immediate centrifugation of cell suspension after the rapid addition of the oxidant mixture to the cell suspension.

All treatments were terminated by centrifugation at 250 g for 5 min at 4°C prior to the subjection of cells to oxidative stress.

#### **4.2.7 Sample preparation**

U937 cell treatments post – oxidation (1 ml, final volume) were stopped by centrifuging at 250 g for 2 min at 4°C in 1.5 ml eppendorf tubes. 0.9 ml of supernatant was carefully

aspirated and discarded. Cell pellets (0.1 ml) were dislodged from the tube walls and resuspended in 5 times volume (0.5 ml) of ice-cold methanol (1: 5, v/v). Samples were stored immediately at  $-70^{\circ}\text{C}$  for a maximum of 2 days prior to FOX analysis.

#### 4.2.8 Enrichment of U937 cells with Fatty acids

Fatty acids (SA, OA, AA, EPA or DHA) were complexed to fatty-acid-free bovine serum albumin (BSA) at a molar ratio of 2.5: 1 and supplemented with culture medium (CM). Fatty acid solutions were complexed to BSA because *in vivo*, these acids are transported in the blood bound to albumin. Ethanolic FA solutions ranging from 2.5 to 10 mM were prepared from a 100 mM stock solution and stored under nitrogen after sterile filtration using nylon syringe filters. FA : BSA complexes were prepared by the addition of an equal volume of 2.5 – 10 mM of FA complexed with 1 – 4 mM of BSA respectively and further diluted with CM to a final volume of 0.9 ml. FA-supplemented culture medium (0.9 ml) was then added to 0.1 ml of cell suspension in each well of a 24 – well plate. Controls were cells incubated with 0.4% (v/v) ethanol only, representing the final ethanol concentration in all FA treatments, which has been shown to have no effect on cell viability as measured by LDH analysis (section 2.3.5.3). Each well contained  $0.5 \times 10^6$  cells/ml. Cells were incubated for 24 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in a humid environment. Final fatty acid concentration ranged from 10 to  $40 \mu\text{M}$ .

Cells were also incubated with  $40 \mu\text{M}$  of each of the fatty acid: SA, OA, AA, EPA or DHA or ethanol only (controls) complexed to BSA at a molar ratio 2.5: 1. Incubations were performed in 24-well plates at  $0.5 \times 10^6$  cells/ml.

For all experiments, after 24 h of FA treatments, cells were washed twice in HBSS by centrifuging at 250 g for 5 min at  $4^{\circ}\text{C}$  and resuspended in  $100 \mu\text{l}$  of HBSS. Cell

suspensions were then transferred from 1.5 ml eppendorf tubes into new and sterile 24-well plates. 900  $\mu$ l of HBSS only (without oxidants) or oxidant mixture in HBSS (freshly prepared from 2 mM t-BHP and 5 mM FeSO<sub>4</sub> stock solutions, just before use) were carefully mixed with cell suspensions and incubated at 37°C and 5% CO<sub>2</sub> for 30 min in a humid environment before sample preparation and FOX analysis. The final oxidant concentration in each well was 750: 750  $\mu$ M of t-BHP: FeSO<sub>4</sub>. All reactions were terminated by centrifugation at 1000 g at 4°C for 2 min. Controls represent cells incubated with 0.4% (v/v) ethanol with or without the subsection to oxidative stress, as indicated.

#### **4.2.9 Supplementation of ascorbic acid, $\alpha$ -tocopherol, $\beta$ -carotene and astaxanthin with the culture medium of U937 cells**

##### ***4.2.9.1 With increasing antioxidant concentrations***

100 mM stock solutions of ascorbic acid in water,  $\alpha$ -tocopherol in ethanol,  $\beta$ -carotene and astaxanthin in chloroform were prepared. Ascorbic acid and  $\alpha$ -tocopherol were stored at 2 – 8°C, while  $\beta$ -carotene and astaxanthin at – 20°C. 4 – 16 mM ascorbic acid, 10 – 40 mM  $\alpha$ -tocopherol and 3 – 12 mM  $\beta$ -carotene and astaxanthin were prepared by serial dilution. Culture medium (CM) was supplemented with aliquots of 4 – 16 mM ascorbic acid, 10 – 40 mM  $\alpha$ -tocopherol and 3 – 12 mM  $\beta$ -carotene or astaxanthin, all to a final volume of 0.9 ml. Antioxidant supplemented CM (0.9 ml), was added to each well of a 24 – well plate containing 0.1 ml of cell suspension after appropriate treatments in the case of the carotenoids where 0.9 ml of carotenoid – supplemented CM was sonicated for 5 s in an ice-bath and sterile – filtered using nylon syringe filters. Each well contained  $0.5 \times 10^6$  cells/ml. Final concentrations of antioxidants varied from 0 – 400  $\mu$ M for ascorbic acid, 0 – 40  $\mu$ M for  $\alpha$ -tocopherol and 0 – 12  $\mu$ M for  $\beta$ -carotene or astaxanthin. Antioxidant-supplemented CM cultured cells were incubated at 37 °C

and 5% CO<sub>2</sub> in a humid environment for 10 min prior to the addition of 40 µM AA, EPA or DHA. Aliquots of 10 mM PUFAs (AA, EPA and DHA) were complexed with 4 mM BSA (as performed earlier) and then added to the cell suspensions. The final volume of cell suspension/well was 1 ml. Plates were then incubated for a further 24 h in conditions stated above. FA-untreated controls were cells incubated with 0.4% (v/v) of ethanol only and FA-treated controls with 40 µM PUFA, both without antioxidant supplementation. For all experiments with antioxidants (single or mixture), both controls were subjected to oxidation as well as the other treatments.

#### ***4.2.9.2 With individual antioxidants***

Cells were incubated with the highest concentration of antioxidants used above (400 µM ascorbic acid, 40 µM α-tocopherol, 12 µM β-carotene or astaxanthin) and 40 µM levels of the PUFAs AA, EPA or DHA following the protocols described above and incubated for a further 24 h at 37°C and 5% CO<sub>2</sub> in a humid environment. Each well contained 0.5 x 10<sup>6</sup> cells/ml.

#### ***4.2.9.3 With antioxidant mixtures***

Cells were also incubated with a mixture of antioxidants for 10 min before CM supplementation with 40 µM PUFAs (AA, EPA or DHA) as described earlier. Controls were U937 cells incubated with 40 µM fatty acid only for 24 h without antioxidant supplementation. Final antioxidant concentrations used were 400 µM ascorbic acid, 40 µM α-tocopherol, 12 µM β-carotene and 12 µM astaxanthin.

For these sets of experiments with antioxidants, cells were washed twice in HBSS after 24 h of treatments, by centrifuging at 250 g for 5 min at 4°C and re-suspended in 100 µl of HBSS. Cell suspensions were then transferred from 1.5 ml eppendorf tubes into new

24-well plates for oxidation experiments. 900  $\mu$ l of oxidant mixture in HBSS (prepared from 2 mM t-BHP and 5 mM FeSO<sub>4</sub> just before use) were carefully mixed with cells suspensions and incubated at 37°C and 5% CO<sub>2</sub> for 30 min before sample preparation and FOX analysis. All reactions were terminated by centrifugation at 1000 g for 2 min at 4°C.

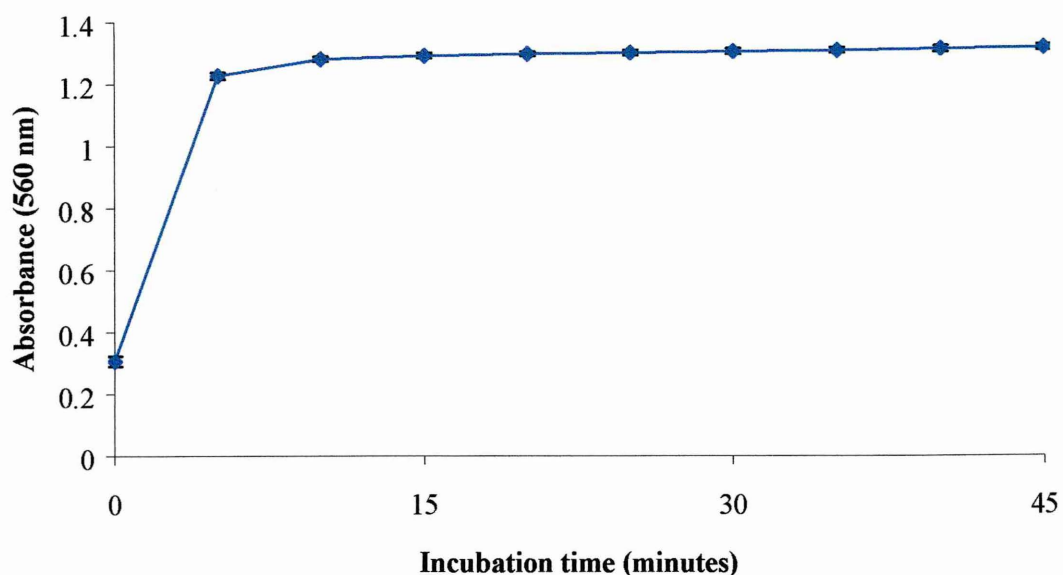
For all experiments with single antioxidants, both FA-untreated and FA-treated controls were subjected to oxidation with 750: 750  $\mu$ M t-BHP: FeSO<sub>4</sub> as well as the other treatments. Likewise, in the case of antioxidant mixtures, controls were also oxidant-stressed with the same radical system.

## **4.3 RESULTS**

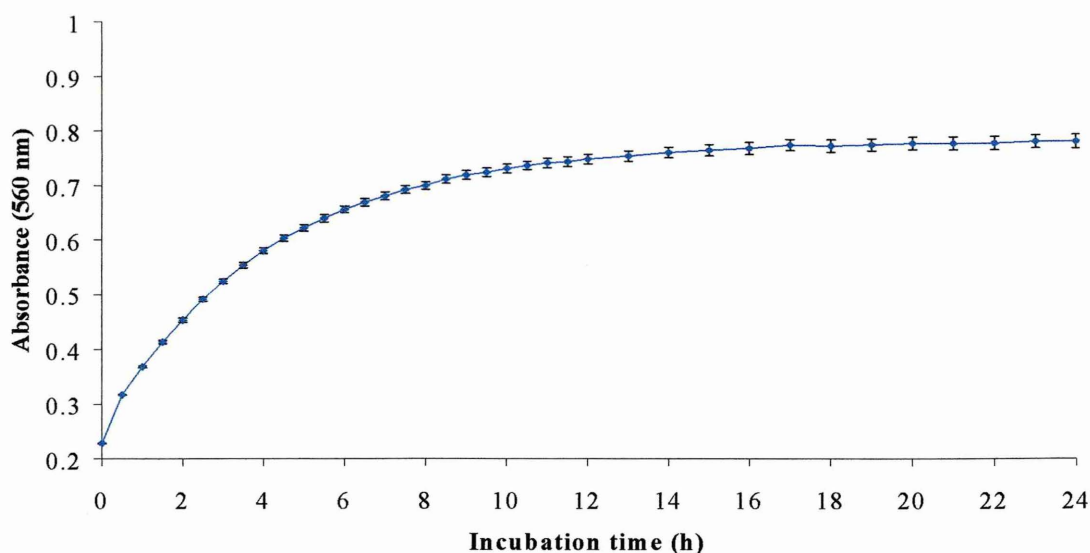
### **4.3.1 Maximal incubation time for the formation of xlenol complex**

Initial studies involved the use of hydroperoxide standards instead of the tissue extracts to study the mechanism of the formation of Fe(III)-xlenol orange complex. This complex is formed by a reaction of H<sub>2</sub>O<sub>2</sub> or the oxidation mixture (t-BHP + FeSO<sub>4</sub>) with Fe<sup>2+</sup> in the presence of xlenol orange. Hydrogen peroxide was incubated with FOX reagent for 45 minutes at room temperature with readings at 560 nm, taken every 5 min (Fig. 4.1). The colour obtained was stable overnight at room temperature. With H<sub>2</sub>O<sub>2</sub>, the reaction was complete in 15 minutes (One-way ANOVA:  $F = 3738.71$ ,  $P < 0.01$ ,  $DF = 9$ ). Tukey's pairwise comparison showed no significant increase in absorbance readings at 560 nm at times  $\geq 15$  min. of incubation performed at room temperature.





**Fig. 4.1** Colour development at room temperature using the FOX reagent with 30  $\mu\text{M}$   $\text{H}_2\text{O}_2$  standard. Means  $\pm$  95% CIs of 2 independent experiments ( $n = 3$ ).



**Fig. 4.2** FOX assay with standard 30: 30  $\mu\text{M}$  t-BHP:  $\text{FeSO}_4$  at room temperature over 24 h. Means  $\pm$  95% CIs of three independent experiments ( $n = 4$ ).

The maximal incubation time required for the formation of Fe (III)-xylenol orange complex with the standard oxidant mixture of t-BHP and  $\text{FeSO}_4$ , was determined by incubating the oxidants at a concentration of 30 : 30  $\mu\text{M}$  with the FOX reagent. Absorbance was then measured at 560 nm over 24 h (Fig. 4.2). The reaction was

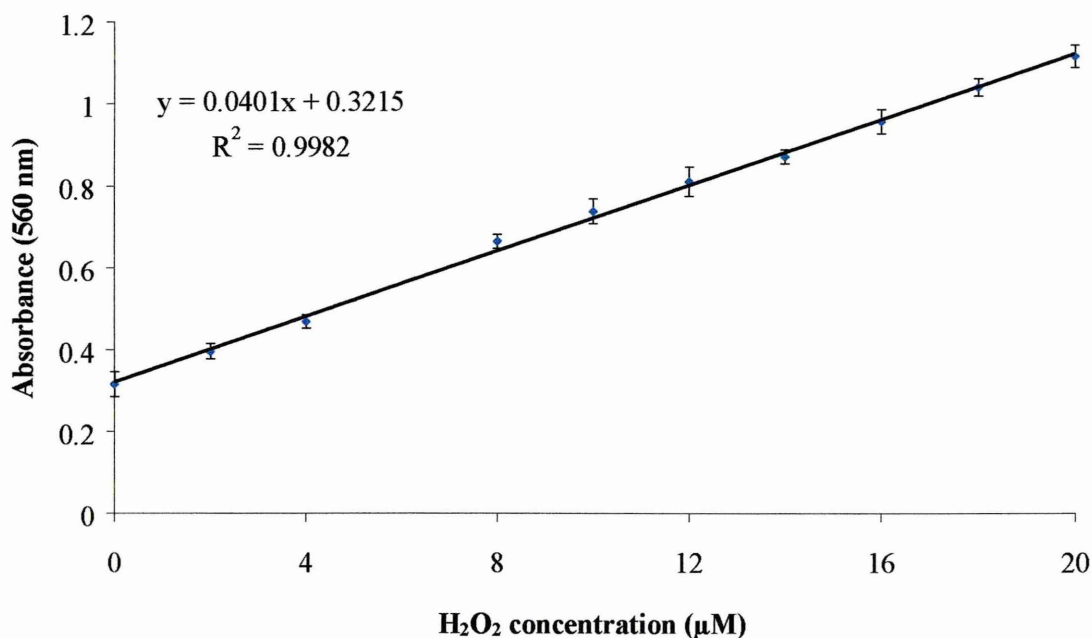
complete in 12 h (One-way ANOVA  $F = 593.84$ ,  $P < 0.01$ ,  $DF = 36$ ). Tukey's pairwise comparisons showed no significant increase in absorbance values at incubation times  $\geq 12$  h at room temperature.

Hydrogen peroxide and t-BHP plus  $\text{FeSO}_4$  were used for standard peroxide measurements for the determination of the maximal incubation time required prior to absorbance readings.

### 4.3.2 Standard curve of hydroperoxides

#### 4.3.2.1 With $\text{H}_2\text{O}_2$

Reactivity of hydrogen peroxide as a standard hydroperoxide with FOX reagent was tested after 30 min of incubation with the FOX reagent at room temperature.



**Fig. 4.3** Standard curve of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in the 0 – 20  $\mu\text{M}$  range. Peroxides were measured at 560 nm with the FOX assay. Means  $\pm$  95% CIs of two independent experiments ( $n = 3$ ).

The data plotted in Fig. 4.3 fit a simple linear model ( $R^2 = 0.998$ ,  $P < 0.01$ ). As expected there is a linear increase in absorbance monitored at 560 nm with increasing  $H_2O_2$  concentration.

#### 4.3.2.2 With iron (II) sulphate $FeSO_4$

In order to establish that standard  $FeSO_4$  on its own does not contribute to the colour development observed with the oxidant mixture of t-BHP:  $FeSO_4$  and the FOX reagent, the FOX assay was performed with varying concentrations of  $FeSO_4$  (Table 4.1). There was no reaction observed in the assay  $FeSO_4$  concentrations ranging from 0 – 1000  $\mu M$  after incubations for 30 min at room temperature (Table 4.1). Results shown in Table 4.1 confirm that  $Fe^{2+}$  must be oxidized by peroxides to  $Fe^{3+}$  to obtain the Fe (III)-xylenol orange complex.

**Table 4.1** Reactivity of iron (II) sulphate ( $FeSO_4$ ) in the FOX assay.

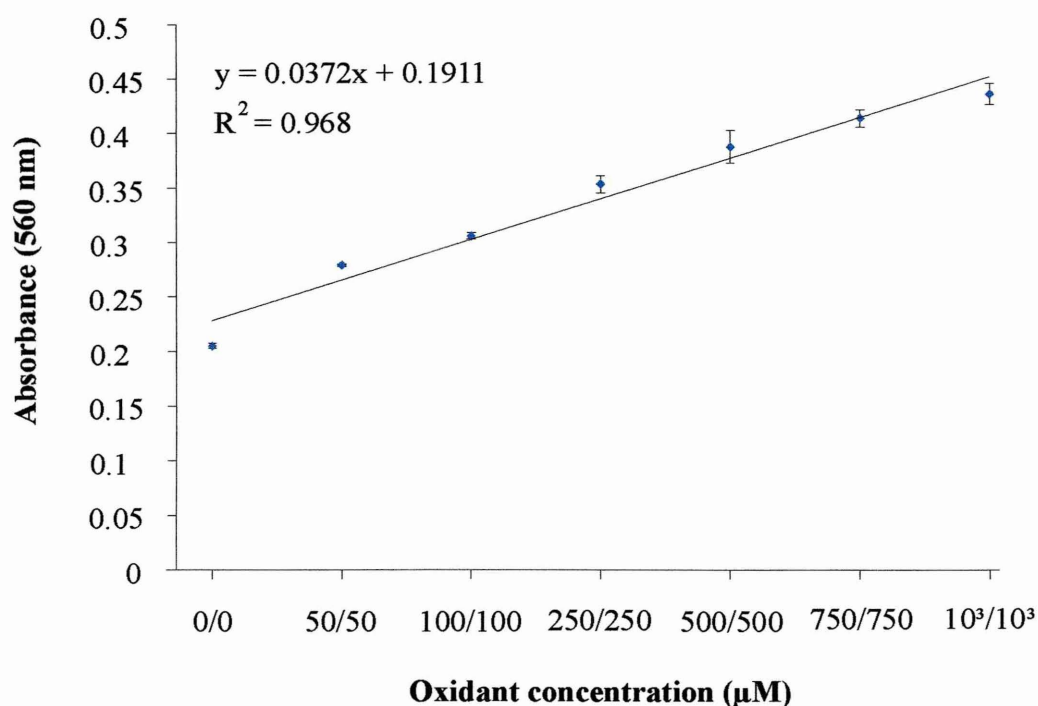
Means  $\pm$  95% CIs of duplicate experiments (n = 4).

[Iron sulphate]	Absorbance at 560 nm
0 $\mu M$	0.1475 $\pm$ 0.0044
100 $\mu M$	0.1468 $\pm$ 0.0012
500 $\mu M$	0.1465 $\pm$ 0.0030
1000 $\mu M$	0.1483 $\pm$ 0.0040

Data analysis by one-way ANOVA showed no significant difference between treatments ( $F = 0.21$ ,  $P > 0.05$ ,  $DF = 3$ ).  $Fe(II)$  on its own exhibited no reactivity with xylenol orange in the assay.

#### 4.3.2.3 With the oxidant mixture (t-BHP: FeSO<sub>4</sub>)

To obtain a standard curve with the oxidant mixture, a 1:1 molar ratio of t-BHP: FeSO<sub>4</sub> with varying concentrations from 0 – 1000  $\mu$ M levels was incubated at room temperature overnight with the FOX reagent prior to the measurements of absorbance (Fig. 4.4). There was a linear increase in absorbance monitored at 560 nm with increasing t-BHP: FeSO<sub>4</sub> levels ( $R^2 = 0.968$ ,  $P < 0.01$ ) confirming the expectation that the greater the concentration of peroxides, the higher the optical density measured with the FOX assay.



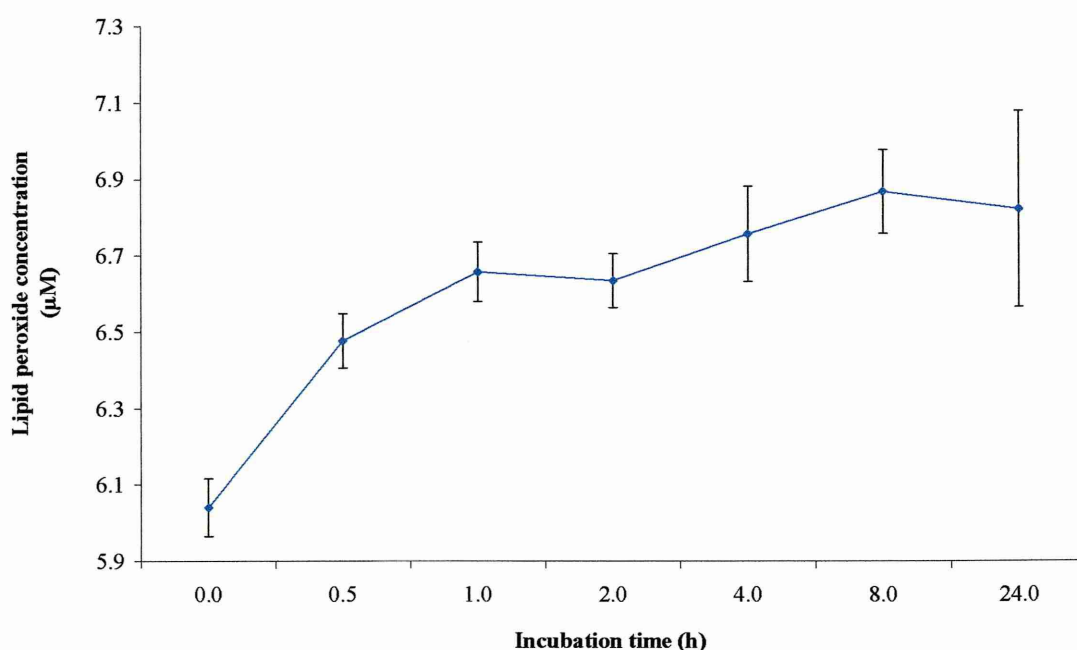
**Fig. 4.4** Standard curve of t-BHP mixture with FeSO<sub>4</sub> in a 1: 1 molar ratio at concentrations from 0 to 1000  $\mu$ M. Data points represent means  $\pm$  CIs of two independent experiments ( $n = 4$ ).

#### 4.3.3 Treatment of cells with oxidants

The next sets of experiments were designed to test that xylenol orange could be used to quantify lipid peroxides in U937 cell extracts.

#### 4.3.3.1 Time-course assay with U937 cells

For a time-dependent effect of the oxidant mixture (t-BHP: FeSO<sub>4</sub>) on the production of lipid hydroperoxides, cells were treated with 750: 750  $\mu$ M oxidant mixture (1:1 molar ratio) for varying incubation times from 0 to 24 h at 37°C and 5% CO<sub>2</sub> in a humid environment (Fig. 4.5).



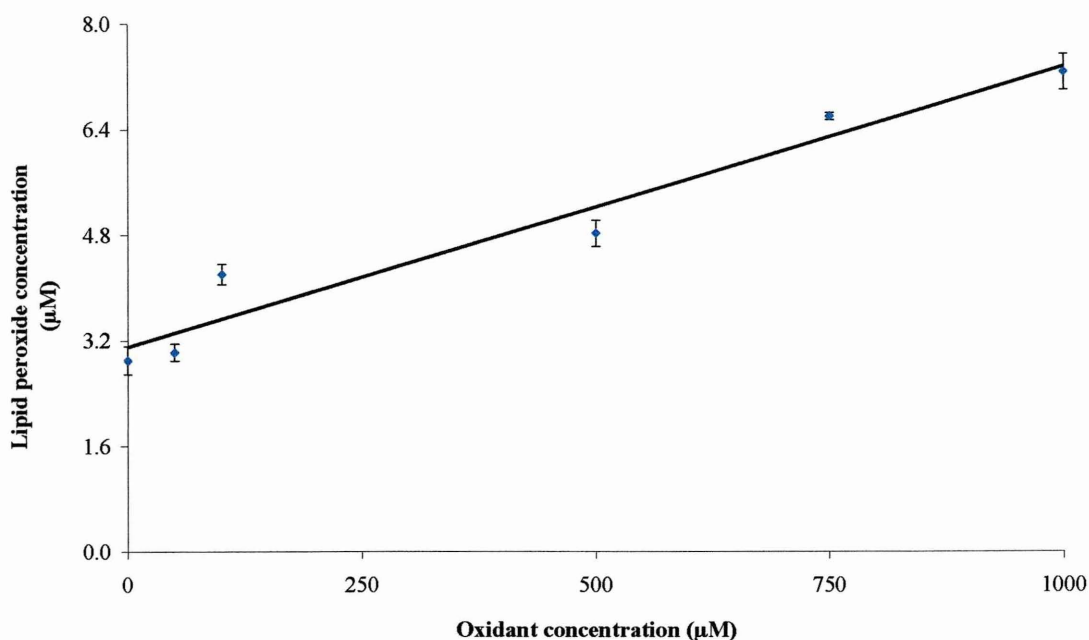
**Fig. 4.5** U937 cells incubated for varying time periods of 0 – 24 h with 750: 750  $\mu$ M of t-BHP: FeSO<sub>4</sub> at 37°C and 5% CO<sub>2</sub> in a humid environment. The amount of Fe(III)-xylenol orange complex formed after overnight incubation at room temperature was measured spectrophotometrically at 560 nm using the EL340 microplate reader. Points represent means  $\pm$  95% CIs from duplicate, independent experiments ( $n = 4$ ). At 0 h, the oxidant mixture was added to the cells and the reaction was stopped by centrifugation immediately after its addition. Apparent extinction coefficient for t-BHP at 560 nm was  $4.3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  (Jiang et al., 1991).

There is a significant increase in the accumulation of lipid peroxides with increasing incubation time of cells (Fig. 4.5) with the radical oxidizing system of t-BHP and FeSO<sub>4</sub>

(One-way ANOVA  $F = 74.00$ ,  $P < 0.01$ ,  $DF = 7$ ). Tukey's comparisons showed a significant difference between cells without oxidant and the 0 h treatment with oxidant. There was a significant increase in lipid peroxidation between the 0 h and 0.5 h treatments. There was no significant difference between results obtained after the incubation of cells with oxidants for 0.5 h and  $\geq 4$  h treatments. At incubation times of 1 h  $\geq 24$  h however, results were not significantly different. A correlation of concentration of lipid peroxides with incubation time (0-24 h) showed an approximately linear response between variables ( $R^2 = 0.81$ ,  $P < 0.01$ ).

#### ***4.3.3.2 Dose-dependent assay of extracts from t-BHP: FeSO<sub>4</sub> treated U937 cells***

For the dose-dependent experiment of cell extracts, the amount of lipid hydroperoxides increased with increasing oxidant concentration ranging from 0/0 to 1000/1000  $\mu\text{M}$  levels of t-BHP/FeSO<sub>4</sub>, as expected. Cells were treated in HBSS with oxidants and incubated for 30 min at 37°C and 5% CO<sub>2</sub> prior to sample preparation for the FOX assay (Fig. 4.6).



**Fig. 4.6** The effects of increasing concentrations of t-BHP:FeSO<sub>4</sub> (1:1 molar concentration) on the reduction of Fe (III)-xylenol orange complex. Means  $\pm$  95% CIs of two independent experiments (n = 4).

Statistical analysis showed a significant increase in the amount of lipid hydroperoxides with increasing oxidant concentration (One-way ANOVA  $F = 378.56$ ,  $P < 0.01$ ,  $DF = 5$ ). Tukey's pairwise comparison failed to show any significant difference between controls (0/0  $\mu\text{M}$ ) and 50/50  $\mu\text{M}$  of oxidant treatment after 30 min of incubation with cells at 37°C and 5%CO<sub>2</sub> in a humid environment. However, there was a significant increase in the amount of lipid hydroperoxides obtained between each of the other treatments. A linear relationship was observed with increasing oxidant concentration in U937 cells oxidised for 30 min ( $R^2 = 0.973$ ,  $P < 0.01$ ).

#### 4.3.4 Fatty acid treatments

##### 4.3.4.1 With Oxidation

In order to examine whether the amount of lipid hydroperoxide produced increases with rising fatty acid levels, cells were treated with 0 – 40  $\mu\text{M}$  FA for 24 h prior to oxidation with 750: 750  $\mu\text{M}$  of t-BHP:  $\text{FeSO}_4$  (Tables 4.2 and 4.3). Pre-oxidation, cells were washed twice in HBSS (4.2.8).

**Table 4.2** Effects of 0 to 40  $\mu\text{M}$  SA and OA treatments on the formation of lipid hydroperoxides. Means of lipid peroxide concentration ( $\mu\text{M}$ )  $\pm$  95% CIs of two independent experiments ( $n = 4$ ). Controls were treated with 0.4% v/v ethanol for 24 h.

Concentration	SA	OA
Unoxidized control	$2.6395 \pm 0.0360$	$2.7209 \pm 0.0335$
Oxidized control	$7.1337 \pm 0.0441$	$9.1453 \pm 0.1000$
10 $\mu\text{M}$	$7.1337 \pm 0.0301$	$9.2413 \pm 0.0365$
20 $\mu\text{M}$	$7.1279 \pm 0.0603$	$9.2238 \pm 0.0834$
30 $\mu\text{M}$	$7.1657 \pm 0.0377$	$9.2093 \pm 0.0811$
40 $\mu\text{M}$	$7.1715 \pm 0.0253$	$9.2180 \pm 0.0459$



**Table 4.3** Effects of PUFA treatments on the formation of lipid hydroperoxides. Means ( $\mu\text{M}$ )  $\pm$  95% CIs of two independent experiments (n = 4).

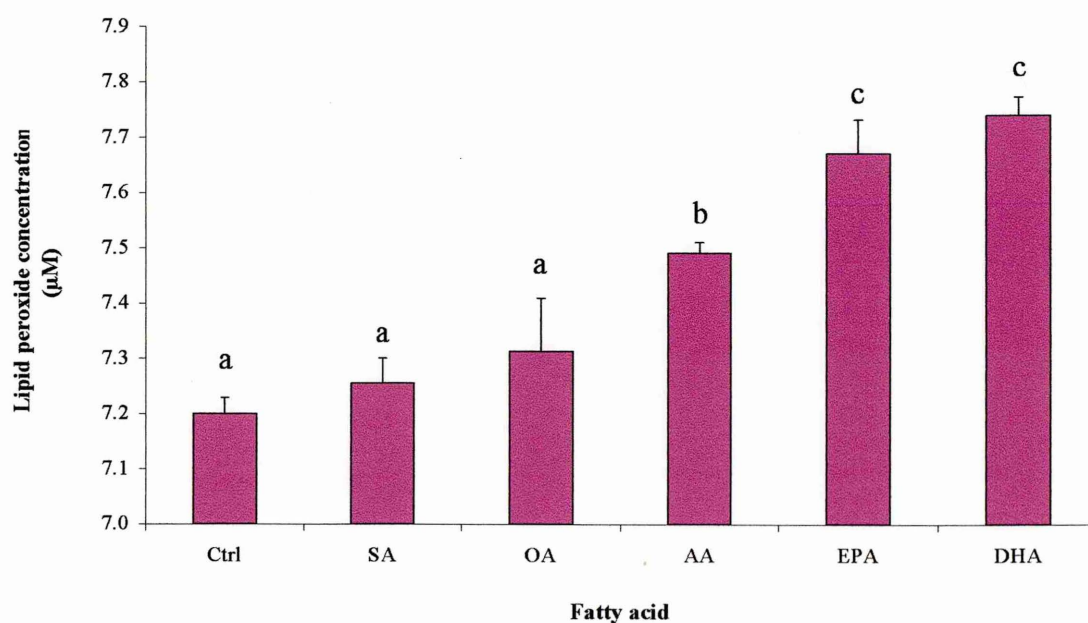
Concentration	AA	EPA	DHA
Unoxidized control	2.7064 $\pm$ 0.0440	2.6192 $\pm$ 0.0781	2.7151 $\pm$ 0.0389
Oxidized control	6.8052 $\pm$ 0.0285	8.0581 $\pm$ 0.0246	8.1047 $\pm$ 0.0161
10 $\mu\text{M}$	6.8198 $\pm$ 0.0237	8.0727 $\pm$ 0.0420	8.0959 $\pm$ 0.0365
20 $\mu\text{M}$	6.8692 $\pm$ 0.0388	8.1192 $\pm$ 0.0410	8.1366 $\pm$ 0.0504
30 $\mu\text{M}$	6.8663 $\pm$ 0.0197	8.2006 $\pm$ 0.0440	8.2248 $\pm$ 0.0675
40 $\mu\text{M}$	6.9797 $\pm$ 0.0459	8.2442 $\pm$ 0.0208	8.2267 $\pm$ 0.0400

In tables 4.2 and 4.3, controls represent cells incubated with ethanol (0.4 %, v/v instead of the fatty acid) with (oxidized control) or without (unoxidized control) oxidant mixtures. There was no significant increase in FOX reactive substances (FOXRS) with increasing SA (One-way ANOVA  $F = 0.94$ ,  $P > 0.05$ ,  $DF = 4$ ) and OA (One-way ANOVA  $F = 0.96$ ,  $P > 0.05$ ,  $DF = 4$ ), varying from 10 – 40  $\mu\text{M}$  for 24 h when compared to oxidized controls. However, there was a marked increase in the formation of lipid peroxides in incubations with the oxidant mixture compared to oxidant-untreated controls (Table 4.2).

Treatment of cells with the polyunsaturated fatty acids AA, EPA and DHA for 24 h prior to oxidation for 30 min (Table 4.3), showed significant increase in the formation of lipid hydroperoxides with increasing FA concentrations. For AA, (One-way ANOVA  $F = 16.73$ ,  $P < 0.01$ ,  $DF = 4$ ); EPA (One-way ANOVA  $F = 19.60$ ,  $P < 0.01$ ,  $DF = 4$ ) and DHA (One-way ANOVA  $F = 6.81$ ,  $P < 0.01$ ,  $DF = 4$ ). In comparison with oxidized controls, there was a significant increase in FOXRS at AA concentrations equal to 40  $\mu\text{M}$ , EPA and DHA levels  $\geq 30 \mu\text{M}$ .

It is noteworthy that comparisons employed in Tables 4.2 and 4.3 were only performed with increasing concentrations of the same FA and not between different FA treatments because experiments were performed on different days, using reagents made up on separate days and cells from different cell populations, thereby generating uncontrolled variations. Hence for these reasons, appropriate controls were included for each set of experiments and thus, in this study, lipid peroxide concentrations were calculated from Beer's law using an apparent extinction coefficient of  $4.3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  for t-BHP (Jiang et al., 1991) at 560 nm.

To investigate the degree of susceptibility of the five different fatty acids to peroxidation, CM was supplemented with 40  $\mu\text{M}$  of each of the five fatty acids complexed with BSA in a 2.5: 1 molar ratio, for 24 h. Cells were then washed twice in HBSS before further incubation for 30 min with 750: 750  $\mu\text{M}$  t-BHP:  $\text{FeSO}_4$  in HBSS (Fig. 4.7).



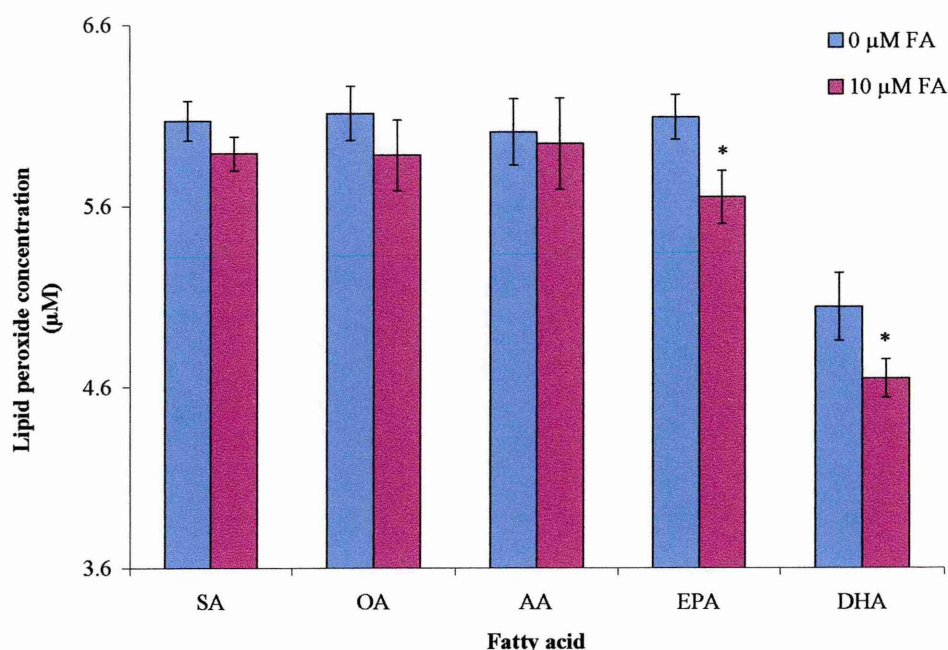
**Fig. 4.7** FOX assay after 24 h treatment of U937 cells with 40  $\mu\text{M}$  of each FA before oxidation for 30 min with t-BHP:  $\text{FeSO}_4$ . Means  $\pm$  95% CIs ( $n = 4$ ). The letters: a, b and c represent significant differences between means at  $P < 0.01$ .

There was a significant difference with fatty acid treatments (One-way ANOVA  $F = 68.12$ ,  $P < 0.01$ ,  $DF = 5$ ). Tukey's pairwise comparisons showed no significant difference between SA and OA compared to controls, confirming the results obtained earlier (Table 4.2). As expected, from results obtained in Table 4.3, there was a marked increase in FOXRS with the PUFAs (AA, EPA and DHA) compared to controls, SA and OA. EPA and DHA treatments yielded results markedly higher than that obtained with AA, although there was no significant increase in FOXRS production between the two treatments (Fig. 4.7). The increase in the accumulation of lipid hydroperoxides with fatty acids suggests that the degree of unsaturation of fatty acid plays a critical role in lipid peroxidation susceptibility of the U937 monocyte cells.

#### **4.3.4.2 Without oxidation**

In order to determine the effects of fatty acid treatments without oxidation, U937 cells were pre-treated with and without  $10\ \mu\text{M}$  levels of the five different fatty acids (SA, OA, AA, EPA and DHA) for 24 h, washed twice in HBSS and re-suspended in HBSS only for 30 min (Fig. 4.8).

In Fig. 4.8, controls represent cells treated with the vehicle only (0.4% v/v, ethanol) for 24 h. There were no significant differences in the accumulation of lipid peroxides after 30 min when compared to their respective controls ( $0\ \mu\text{M}$ ) with SA ( $T = 2.44$ ,  $P > 0.05$ ,  $DF = 6$ ); OA ( $T = 1.86$ ,  $P > 0.05$ ,  $DF = 6$ ) and AA ( $T = 0.35$ ,  $P > 0.05$ ,  $DF = 6$ ). However, in comparison to their controls, the concentration of lipid peroxides decreased significantly with EPA and DHA treatments: EPA ( $T = 4.55$ ,  $P < 0.01$ ,  $DF = 6$ ); DHA ( $T = 3.61$ ,  $P < 0.05$ ,  $DF = 6$ ). With EPA versus control, there was 7.3% decrease and 7.8% with DHA versus control.



**Fig. 4.8** Effect of FA-treatment on U937 cells without exposure to oxidants (in HBSS only for 30 min). Means  $\pm$  95% CI (n = 4). \* Represents significant difference between FA-treated and FA-untreated cells.

Interestingly, EPA and DHA appear to suppress lipid peroxidation without induced-oxidation but produce a reversed effect upon oxidative stress. Taken together, studies show that increasing accumulation of lipid hydroperoxides observed with the PUFAs is as a combined effect of the PUFAs and oxidants, as expected.

#### 4.3.5 With antioxidants

To characterize the antioxidant effects of ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin, U937 cells were incubated with these antioxidants 10 min before 40  $\mu$ M PUFA supplementation. After 24 h, the production of lipid hydroperoxides was monitored spectrophotometrically at 560 nm after the subsection of cells to oxidation for 30 min with 750: 750  $\mu$ M t-BHP: FeSO<sub>4</sub> at 37°C. Antioxidant concentrations chosen were dependent on LDH results obtained in chapter two. Figs. 4.9 to 4.12 show the

effects of varying the concentrations of either the water-soluble (ascorbic acid) or lipid-soluble ( $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin) antioxidants on the formation of lipid hydroperoxides as monitored at 560 nm from U937 cells incubated with oxidants for 30 min at 37°C. Having established the marked difference between FA-untreated cells treated with and without oxidants (Tables 4.2 and 4.3), subsequent experiments do not report data for controls with vehicle-treated cells without the oxidant.

**(A) With ascorbic acid**

The effect of the addition of 0 – 400  $\mu$ M ascorbic acid in cells exposed to 750: 750  $\mu$ M t-BHP: FeSO<sub>4</sub> is shown in Fig. 4.9. When ascorbic acid treatments were compared with oxidized FA-treated controls, Tukey's pairwise comparisons showed a significant decrease in the total hydroperoxide formed in U937 cells with 400  $\mu$ M ascorbic acid concentration for all three PUFAs: AA (One-way ANOVA  $F = 38.30$ ,  $P < 0.01$ ,  $DF = 5$ ); EPA (One-way ANOVA  $F = 78.88$ ,  $P < 0.01$ ,  $DF = 5$ ); DHA (One-way ANOVA  $F = 59.83$ ,  $P < 0.01$ ,  $DF = 5$ ). The presence of AA, EPA or DHA in U937 cells gave significantly higher lipid hydroperoxide values than incubations without PUFA supplementation.

**(B) With  $\alpha$ -tocopherol**

Methanolic-extracts from cells incubated with 0 – 40  $\mu$ M  $\alpha$ -tocopherol for 24 h were analysed for lipid hydroperoxide using the colorimetric assay based on Fe (III)-xylenol orange complex formation post oxidation with 750: 750  $\mu$ M t-BHP: FeSO<sub>4</sub> for 30 min (Fig. 4.10). Co-treatment with AA, significantly suppressed the formation of lipid hydroperoxides at  $\alpha$ -tocopherol  $\geq 10$   $\mu$ M below that obtained with oxidized FA-treated controls (One-way ANOVA  $F = 9.04$ ,  $P < 0.01$ ,  $DF = 5$ ). In fact, there was no significant difference between unoxidized FA-untreated controls and  $\alpha$ -tocopherol

levels  $\geq 10 \mu\text{M}$ . At  $40 \mu\text{M}$   $\alpha$ -tocopherol, there was a significant decrease by 7.5 % in the accumulation of FOXRS compared to unoxidized FA-treated controls. With EPA (One-way ANOVA  $F = 7.13$ ,  $P < 0.01$ ,  $DF = 5$ ), there was no significant difference between unoxidized FA-untreated controls and  $10 - 40 \mu\text{M}$   $\alpha$ -tocopherol treatments. Similarly, in incubations containing DHA (One-way ANOVA  $F = 5.29$ ,  $P < 0.01$ ,  $DF = 5$ ), Tukey's pairwise comparisons showed a significant reduction in the formation of lipid hydroperoxides at  $\alpha$ -tocopherol concentrations  $> 10 \mu\text{M}$  when compared to oxidized FA-treated controls. The dose-dependent decrease in the accumulation of FOXRS observed in extracts with increasing  $\alpha$ -tocopherol levels as monitored at 560 nm was, however, not significantly different between the concentrations used.

### (C) *With $\beta$ -carotene*

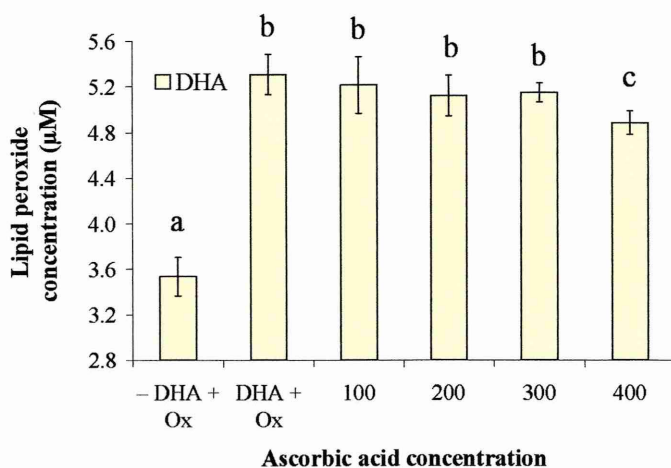
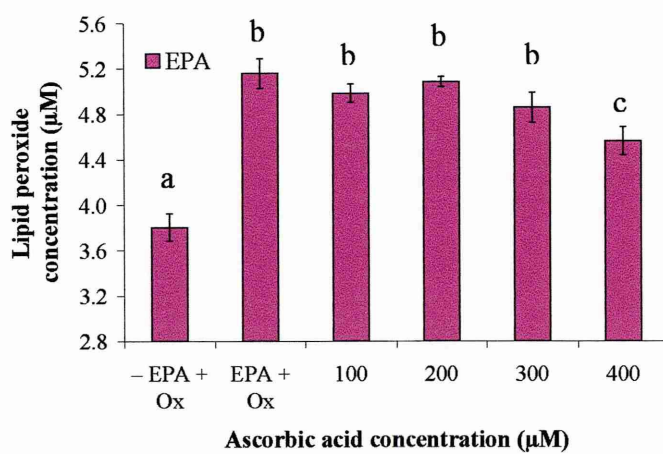
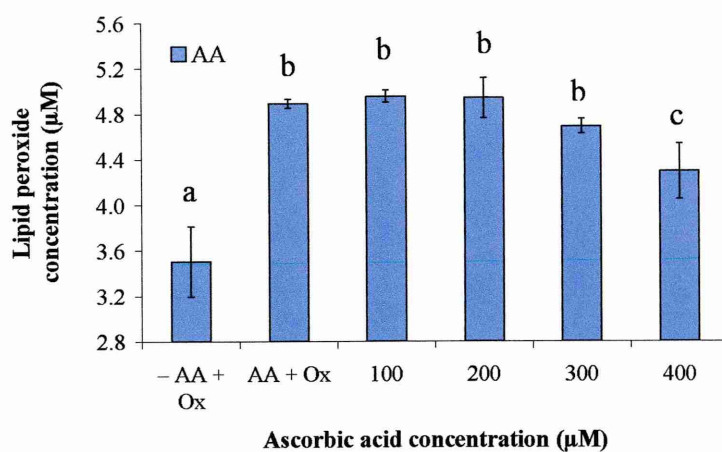
In order to test that the pre-treatment of U937 cells with  $\beta$ -carotene for 24 h induces potent antioxidant effects, cells response to oxidation with 750: 750  $\mu\text{M}$  t-BHP:  $\text{FeSO}_4$  for 30 min was assessed for the production of lipid peroxides by the FOX assay. As shown in Fig. 4.11, treatment of cells with  $3 - 12 \mu\text{M}$   $\beta$ -carotene led to a decrease in the amount of lipid hydroperoxides generated. With  $40 \mu\text{M}$  AA (One-way ANOVA  $F = 41.05$ ,  $P < 0.01$ ,  $DF = 5$ ), Tukey's pairwise comparisons showed a concentration-dependent decrease in lipid peroxidation with increasing  $\beta$ -carotene levels, significantly different at concentrations  $> 9 \mu\text{M}$  when compared with oxidized FA-treated controls. At  $12 \mu\text{M}$   $\beta$ -carotene there was a 15.8 % decrease in the concentration of FOXRS when compared to unoxidised FA-treated controls and this was significant. With  $40 \mu\text{M}$  EPA (One-way ANOVA  $F = 4.88$ ,  $P < 0.05$ ,  $DF = 5$ ), a significant decrease in lipid peroxidation was observed with  $12 \mu\text{M}$   $\beta$ -carotene treatment, compared to oxidized FA-treated controls. Experiments with  $40 \mu\text{M}$  DHA (One-way ANOVA  $F = 18.02$ ,  $P <$

0.01, DF = 5), produced a dose-dependent decrease in the accumulation of lipid hydroperoxides with increasing  $\beta$ -carotene, significant at 12  $\mu$ M levels.

**(D) With astaxanthin**

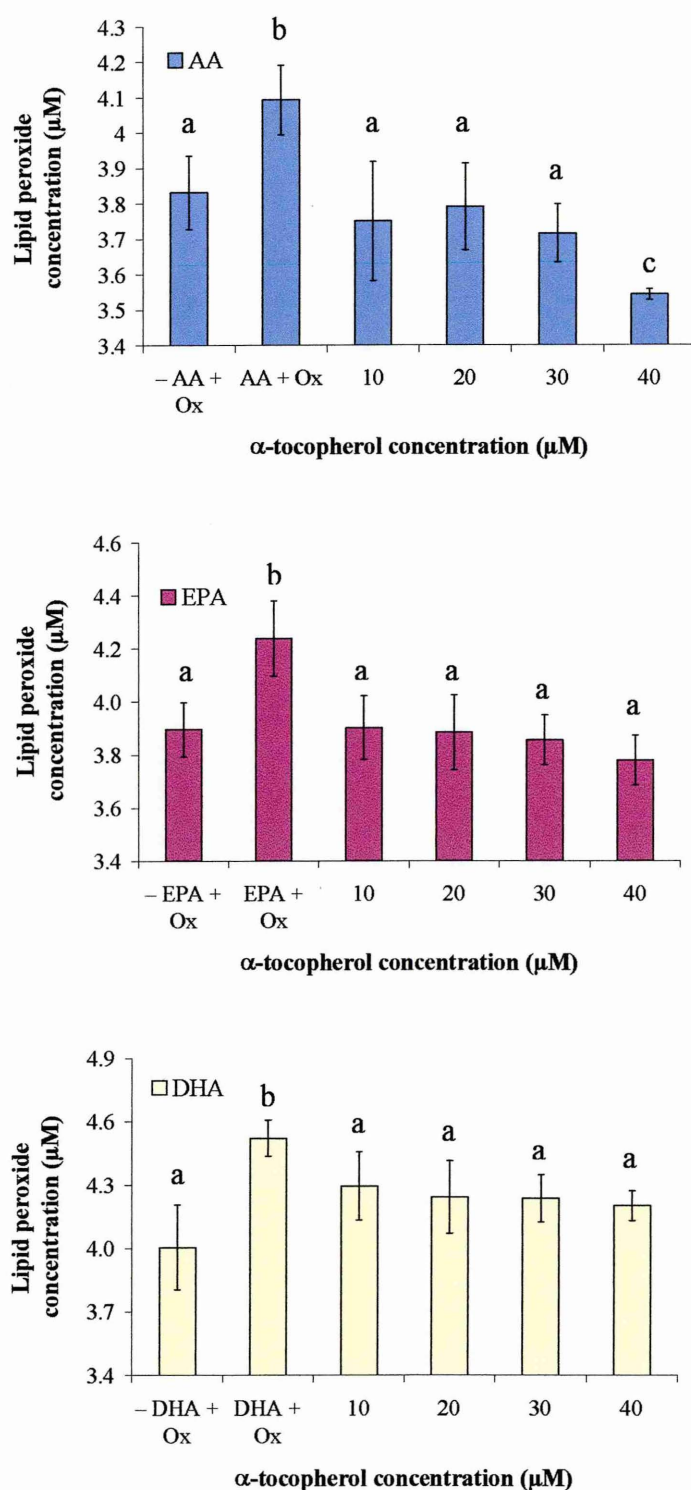
To examine whether the presence of 0 – 12  $\mu$ M astaxanthin conferred protection against oxidative stress induced by 750: 750  $\mu$ M t-BHP: FeSO<sub>4</sub>, U937 cells were pre-treated with astaxanthin and PUFAs for 24 h prior to oxidation for 30 min. Fig. 4.12 shows astaxanthin as a potent antioxidant at 12  $\mu$ M when compared to oxidized FA-treated controls. With AA (One-way ANOVA F = 43.72, P < 0.01, DF = 5), there was a significant decrease in lipid peroxidation at 12  $\mu$ M astaxanthin compared to unoxidized FA-treated controls. Similarly, with EPA (One-way ANOVA F = 16.99, P < 0.01, DF = 5) and DHA (One-way ANOVA F = 6.26, P < 0.01, DF = 5) there was a significant reduction in induced peroxidation at 12  $\mu$ M astaxanthin concentrations.

For all experiments with antioxidants, there was a significant increase in lipid peroxidation with oxidized FA-treated controls when compared with unoxidized FA-treated controls.

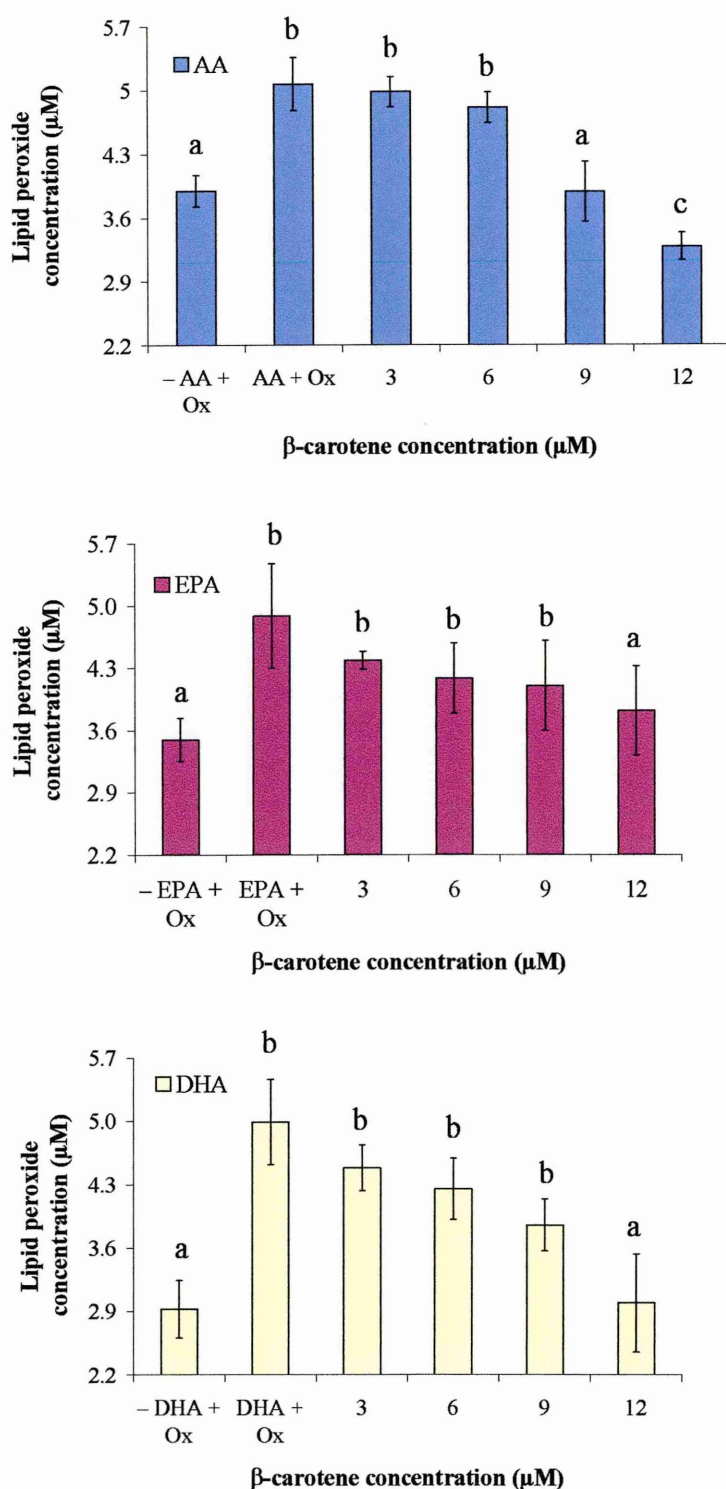


**Fig. 4.9** Antioxidant effects of 0 – 400 μM ascorbic acid in U937 cells enriched for 24 h with 40 μM AA, EPA or DHA. Means ± 95% CIs of two independent experiments. For AA (n = 3); EPA and DHA (n = 4). The letters: a, b and c denote significant differences between means at  $P < 0.01$  by one-way ANOVA.

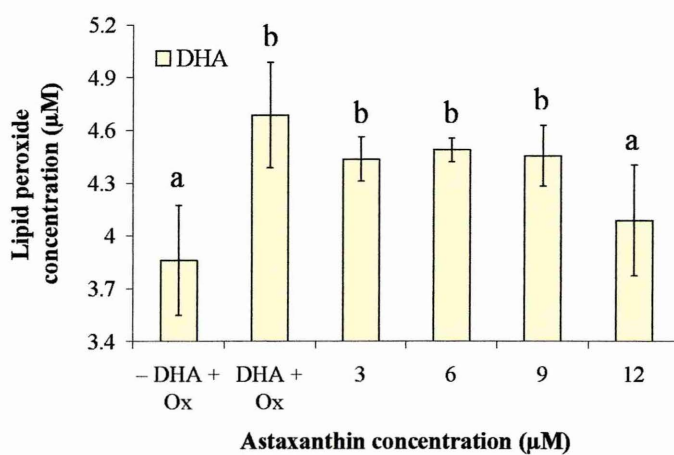
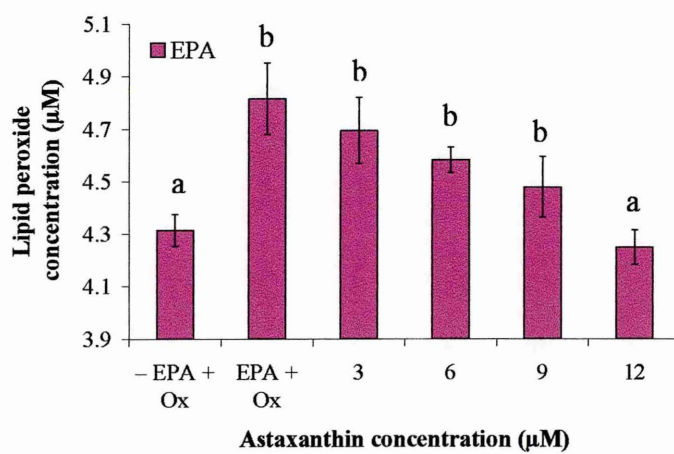
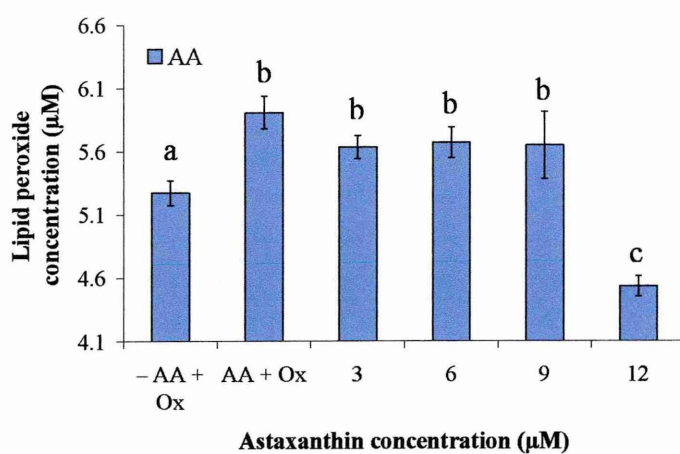




**Fig. 4.10**  $\alpha$ -tocopherol (0 – 40  $\mu$ M) antioxidant activity in U937 cells cultured in CM-supplemented with 40  $\mu$ M AA, EPA or DHA for 24 h at 37°C and 5% CO<sub>2</sub> in a humid environment. Values represent means  $\pm$  CIs from two independent experiments (n = 4). The letters a, b and c indicate significant differences between treatments at  $P < 0.01$  by one-way ANOVA.



**Fig. 4.11** Dose-dependent effect of 0-12  $\mu\text{M}$   $\beta$ -carotene on oxidative stress induced by 750: 750  $\mu\text{M}$  t-BHP:  $\text{FeSO}_4$  oxidising system for 30 min after 24 h of 40  $\mu\text{M}$  PUFA (AA, EPA or DHA) supplementation. Means  $\pm$  95% CIs from two independent experiments ( $n = 4$ ). The letters: a, b and c indicate significant differences between treatments at  $P < 0.05$  by one-way ANOVA.

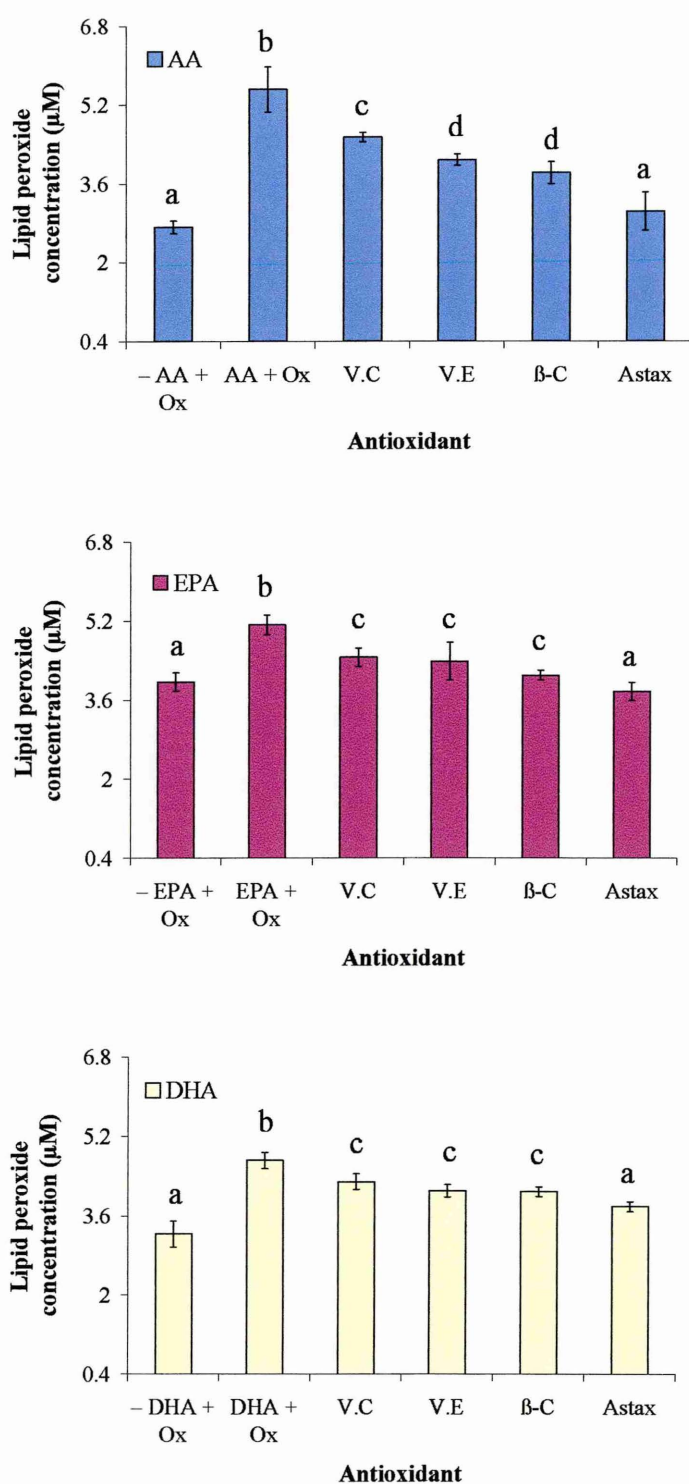


**Fig. 4.12** The inhibition of lipid peroxidation by the xanthophyll, astaxanthin (0 – 12 μM) in U937 cells pre-treated with AA, EPA or DHA for 24 h prior to induced oxidation. Means ± 95% CIs from two independent experiments (n = 4). The letters: a, b and c represent significant differences between treatments at  $P < 0.01$  by one-way ANOVA.

#### *4.3.5.1 Comparison of individual antioxidant effects*

To compare the antioxidant activities of ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin, cells were pre-treated with these antioxidants and AA, EPA or DHA for 24 h, after which they were exposed to 750: 750  $\mu$ M t-BHP: FeSO<sub>4</sub> in HBSS and incubated for 30 min at 37°C and 5% CO<sub>2</sub> in a humid environment. Final antioxidant concentrations used were 400  $\mu$ M ascorbic acid, 40  $\mu$ M  $\alpha$ -tocopherol, 12  $\mu$ M  $\beta$ -carotene and 12  $\mu$ M astaxanthin. The ability of these antioxidants to inhibit lipid oxidation was evaluated with the FOX assay at 560 nm.

The presence of the four antioxidants markedly inhibited the generation of lipid hydroperoxides when U937 cells were oxidatively stressed. The amounts of lipid hydroperoxide produced after the incubation of U937 cells with either 400  $\mu$ M ascorbic acid, 40  $\mu$ M  $\alpha$ -tocopherol, 12  $\mu$ M  $\beta$ -carotene or 12  $\mu$ M astaxanthin coupled with the PUFAs (AA, EPA or DHA) for 24 h before oxidation, were determined (Fig. 4.13). Comparative measurements of antioxidant activity after t-BHP/Fe<sup>2+</sup> induced peroxidation indicated that 12  $\mu$ M astaxanthin was the most powerful antioxidant in all three experiments with AA (One-way ANOVA  $F = 53.14$ ,  $P < 0.01$ ,  $DF = 5$ ); EPA (One-way ANOVA  $F = 17.53$ ,  $P < 0.01$ ,  $DF = 5$ ); or DHA (One-way ANOVA  $F = 36.58$ ,  $P < 0.01$ ,  $DF = 5$ ). All antioxidant treatments gave significantly lower concentrations of FOXRS compared to oxidized FA-treated controls in the following order: ascorbic acid <  $\alpha$ -tocopherol <  $\beta$ -carotene < astaxanthin for all three PUFAs (AA, EPA and DHA).

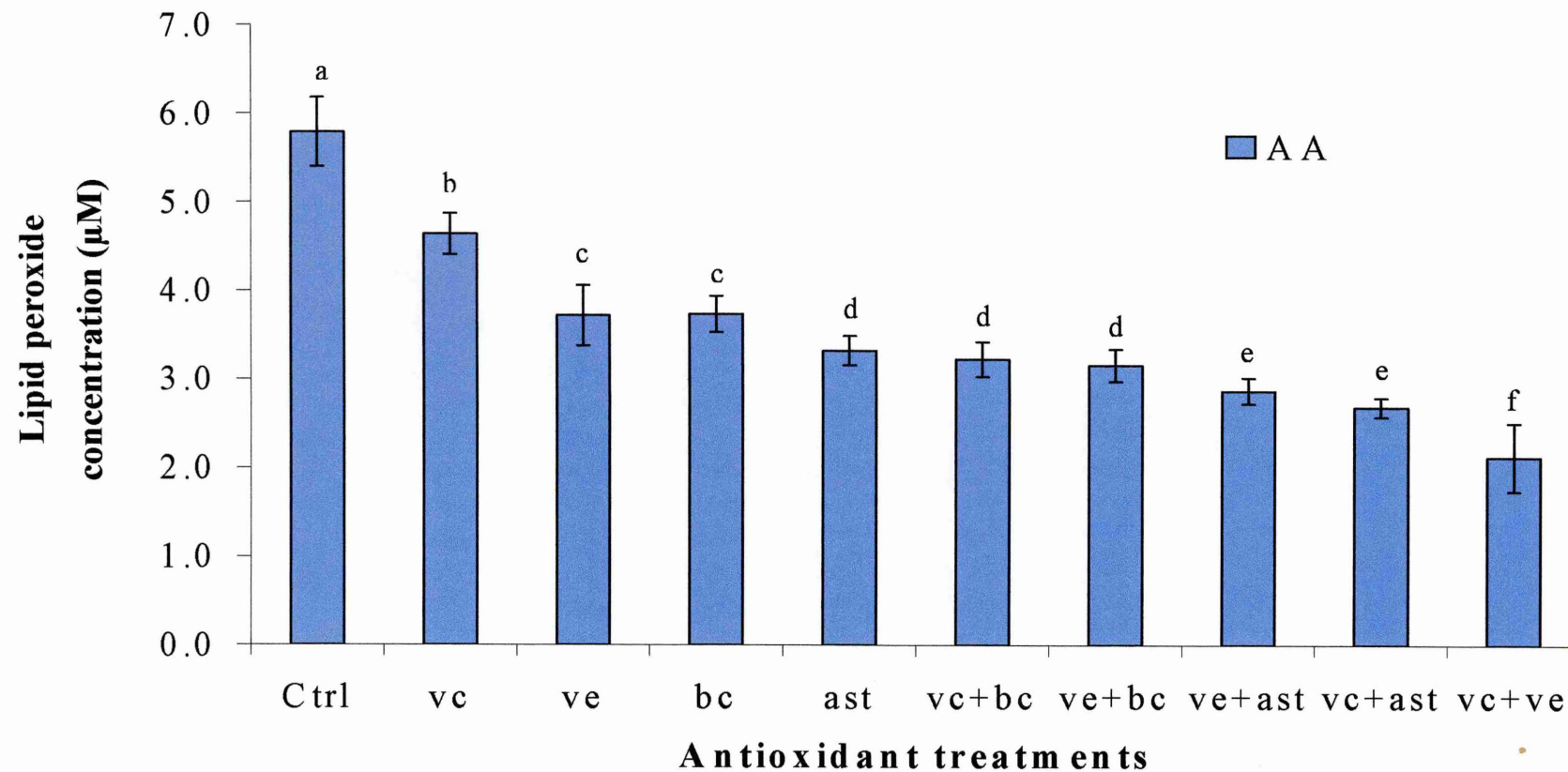


**Fig. 4.13** Comparative studies of the antioxidant activities of ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin in U937 cells pre-treated with 40  $\mu$ M AA, EPA or DHA for 24 h prior to oxidation with 750/750  $\mu$ M of t-BHP/ $\text{Fe}^{2+}$  radical system. The letters: a, b, c and d represent significant differences between treatments at  $P < 0.01$  by one-way ANOVA.

#### 4.3.5.2 With a combination of antioxidants

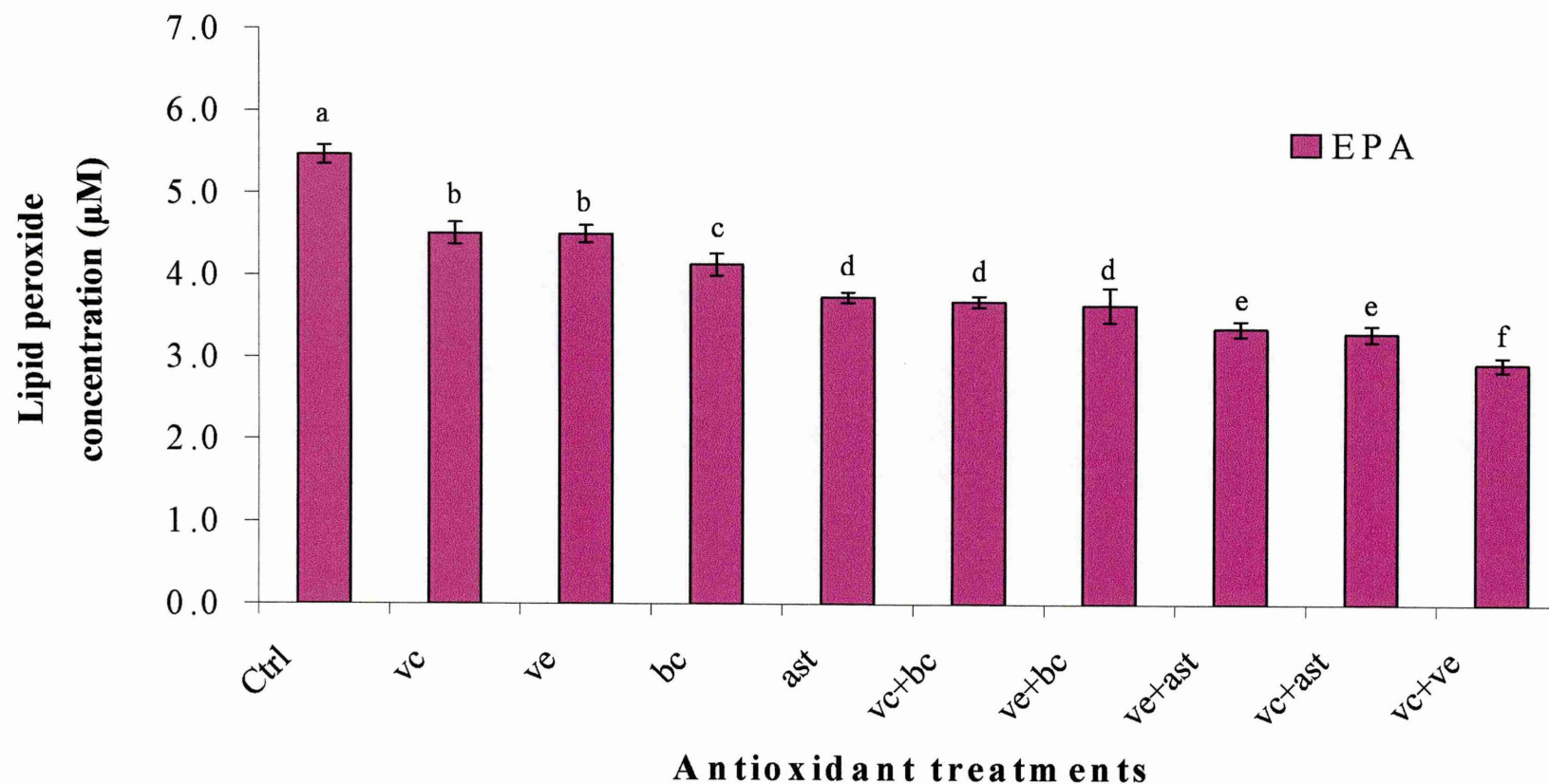
To study the interactive effects between antioxidants, combinations of two different agents were incubated with U937 cells in addition to PUFA (AA, EPA or DHA) for 24 h prior to oxidation (Figs. 4.14 to 4.16). The relationship between optical density and the amount of hydroperoxide produced was evaluated when antioxidant mixtures were incorporated into U937 cells. The most potent combination of antioxidants against lipid peroxidation induced by the radical-mediated system of t-BHP/Fe<sup>2+</sup> in U937 cells was a mixture of ascorbic acid (vc) and  $\alpha$ -tocopherol (ve). For AA (Fig. 4.14) (One-way ANOVA F = 64.76, P < 0.01, DF = 9); EPA (Fig. 4.15) (One-way ANOVA F = 151.96, P < 0.01, DF = 9) and DHA (Fig. 4.16) (One-way ANOVA F = 67.56, P < 0.01, DF = 9).

Charts show the antioxidant power in an ascending order of magnitude (Figs. U-W). With AA, there was a 63% reduction in hydroperoxide yield with a combination of ascorbic acid and  $\alpha$ -tocopherol compared to controls, whereas individually, there was a 19.9% and 35.7% decrease with ascorbic acid and  $\alpha$ -tocopherol respectively suggesting a synergistic activity of these two antioxidants. In the same manner, results from EPA experiments yielded a 46.4% decrease with vc+ve compared to controls, and 17.4% or 17.6% with ascorbic acid or  $\alpha$ -tocopherol respectively. Furthermore, DHA treatments with a mixture of vc+ve resulted in a 47% decrease compared to controls and 16% or 21% decrease with vc (ascorbic acid) or ve ( $\alpha$ -tocopherol) alone respectively. From the three experiments with AA, EPA or DHA, all antioxidant combinations yielded better antioxidant effects than that obtained with individual treatments (Figs. 4.14 to 4.16).



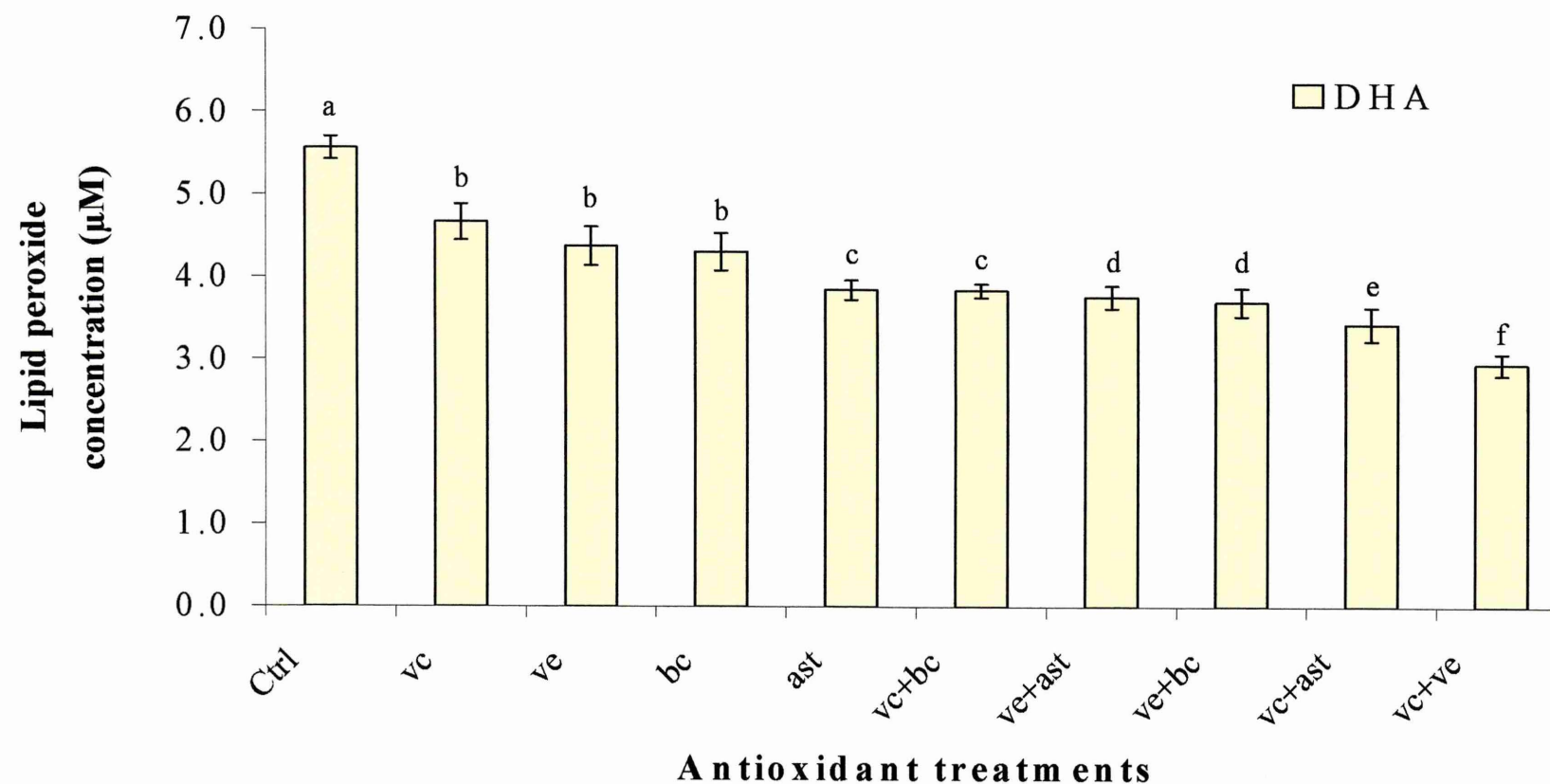
**Fig. 4.14** Effects of the combination of antioxidants on oxidized arachidonic acid (AA) - treated U937 cells. Means  $\pm$  95% CIs (n=4). Controls (Ctrl) involved cells treated with 40  $\mu$ M AA and oxidants. Ctrl represents control, vc, ascorbic acid, ve,  $\alpha$ -tocopherol, bc,  $\beta$ -carotene and ast, astaxanthin. The letters: a, b, c, d, e and f represent significant differences between means at  $P < 0.01$ .





**Fig. 4.15** Effects of the combination of antioxidants on oxidized eicosapentaenoic acid (EPA) - treated U937 cells. Means  $\pm$  95% CIs (n =4). Controls (Ctrl) involved cells treated with 40  $\mu$ M EPA and oxidants. Ctrl represents control, vc, ascorbic acid, ve,  $\alpha$ -tocopherol, bc,  $\beta$ -carotene and ast, astaxanthin. Letters: a – e indicate significant differences between means at  $P < 0.01$  (one-way ANOVA).





**Fig. 4.16** Effects of the combination of antioxidants on oxidized docosahexaenoic acid (DHA) - treated U937 cells. Means  $\pm$  95% CIs (n =4). Controls (Ctrl) involved cells treated with 40  $\mu$ M DHA and oxidants. Ctrl represents control, vc, ascorbic acid, ve,  $\alpha$ -tocopherol, bc,  $\beta$ -carotene and ast, astaxanthin. Letters: a – e indicate significant differences between treatments at P < 0.01.

Overall, the fat-soluble antioxidants ( $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin), on their own conferred a better antioxidant protective effects against the formation of lipid hydroperoxides initiated by the radical oxidizing system of 750: 750  $\mu$ M t-BHP:  $\text{FeSO}_4$  than the water-soluble antioxidant (ascorbic acid).

#### 4.4 DISCUSSION AND CONCLUSION

The purpose of this study was to assess the susceptibility of U937 cells to oxidative stress after cellular enrichment with the polyunsaturated fatty acids AA, EPA and DHA for 24 h. A second objective was to determine the effectiveness of the antioxidants: ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin, both individually and as mixtures in the U937 cell model.

In the FOX-modified assay used here to study lipid peroxidation, hydrogen peroxide and tert-butyl hydroperoxide were used as standard peroxides for the determination of the incubation time required for the completion of Fe (III)-xylenol orange complex formation. With  $\text{H}_2\text{O}_2$ , the reaction was complete in 15 min whereas it took 12 h for t-BHP. Since oxidation in U937 cell model was induced with the t-BHP/ $\text{Fe}^{2+}$  system, measurement at 560 nm of Fe (III)-xylenol orange complex formed was performed after incubation at room temperature for 12 – 24 h in the dark, based on the results obtained in Fig. 4.2. A standard curve with increasing concentrations of  $\text{H}_2\text{O}_2$  (Fig. 4.3) or t-BHP/ $\text{Fe}^{2+}$  (Fig. 4.4) gave good positive correlations with absorbance at 560 nm, showing an increase in optical density with increasing hydroperoxide concentration. FOX assay with  $\text{FeSO}_4$  alone yielded no increase in colour intensity therefore, showing that the presence of Fe (II) in the standard oxidant mixture did not contribute to the increasing optical density obtained with t-BHP/ $\text{Fe}^{2+}$  radical system employed.

This research shows that lipid hydroperoxides can be detected after 30 min of incubating U937 cells with 750: 750  $\mu\text{M}$  t-BHP:  $\text{FeSO}_4$  when compared to the 0 h experiment (in which the oxidant mixture was added and the reaction stopped immediately) by the FOX assay (Fig. 4.5). The accumulation of lipid hydroperoxides increased with time. As expected, the concentration of lipid peroxides increased with the exposure of U937 cells to increasing oxidant concentrations (Fig. 4.6). The exposure of U937 cells to t-BHP has previously been reported to give a dose- and time-dependent increase in lipid peroxidation by-products (Nardini et al., 1998; Spickett et al., 2001), and this is supported by the results obtained in the present study. The amount of lipid hydroperoxide produced was found to be dependent on the severity and length of the oxidative treatment. Spickett et al (2001) subjected U937 cells to t-BHP/ $\text{Fe}^{2+}$  levels varying from 0.05 to 1 M t-BHP and 0.01 M  $\text{Fe}^{2+}$  levels to obtain total ion current (TIC) traces by LC-MS to detect lipid oxidation. This suggests that U937 cells are quite resistant to high levels of oxidation over short time courses (Spickett et al., 2001). This is not surprising because these cells are phagocytes, capable of generating oxidants for anti-microbial defence and thus may be expected to possess inherent antioxidant protection.

A concentration of 750: 750  $\mu\text{M}$  t-BHP:  $\text{FeSO}_4$  was chosen based on viability results obtained in chapter two. On the basis that this concentration had a significant effect on LDH leakage in U937 cells compared to controls, the efficacy of antioxidants in affecting the accumulation of lipid hydroperoxides was tested in response to a substantial degree of oxidative stress, induced with the above concentration. From now on, this oxidative condition of t-BHP/ $\text{Fe}^{2+}$  (750/750  $\mu\text{M}$ ) treatment will also be referred to as the oxidant mixture. Alkoxy and peroxy radicals produced by the oxidant mixture initiate lipid peroxidative processes (Latour et al., 1995).

#### 4.4.1 Fatty acid treatments

In comparison to untreated controls, pre-treatment for 24 h with 10  $\mu$ M EPA or DHA significantly reduced the generation of lipid peroxides whilst there were no differences observed with SA, OA or even AA when cells were incubated in HBSS only without oxidation (Fig. 4.8). The notion that the PUFAs purchased from the manufacturer were partially oxidised, as previously suggested in chapter two, is ruled out here. This suggests that the presence of these long chain, highly-unsaturated fatty acids (EPA and DHA) in cell membranes influences a number of membrane functions including affecting membrane permeability to electrolytes and non-electrolytes (Williams and Hazel, 1993) present in the surrounding environment (CM) some of which may induce lipid peroxidative damage, under normal conditions without oxidation. On the other hand, the incubation of U937 cells with SA or OA showed no significant difference in lipid peroxidation compared to controls when challenged with oxidants. However, enrichment with equal amounts of PUFAs (AA, EPA and DHA) increased the susceptibility of these cells to peroxidative damage, which increased linearly with the degree of unsaturation (Fig. 4.7). This is consistent with the study by Maziere et al (1998) who reported elevations in intracellular lipid peroxidation products in low-density lipoproteins. Hsu et al. (2000) found that cultured U937 cells mainly contained fatty acids with 14 – 18 carbon atoms and PUFAs such as AA, present only as minor components. It can therefore be said that the treatment of U937 cells with PUFAs AA, EPA and DHA makes the cells more susceptible to oxidative damage, in terms of the content of lipid hydroperoxides produced.

This study demonstrates that under equal conditions of oxidative stress, fatty acids oxidize at different rates and produce lipid hydroperoxides in a manner related to their degree of unsaturation. In agreement with this study is the finding that the production

of lipid peroxides, as evaluated by an iodometric method, was higher with the PUFAs (AA, EPA and DHA) in comparison to SA and OA (Visioli et al., 1998). However, that study reported lower peroxide levels with EPA compared to AA and DHA when oxidation was stimulated with 2, 2'-azo-bis-(2-amidinopropane) dihydrochloride (AAPH) in non-esterified fatty acid micelles *in vitro*. In contrast, an increase in the formation of lipid hydroperoxides was found with increasing fatty acid unsaturation using U937 cell models (Fig. 4.7). The results obtained by Visioli et al (1998) may have been due to the system employed because fatty acids in biological cell membranes are present in esterified forms in triacylglycerols, phospholipids and cholesterol thus affecting their susceptibility to oxidative stress.

This study clearly demonstrates that U937 cells pre-cultured with PUFA of the n-6 (AA) and n-3 (EPA and DHA) series are more susceptible to peroxidative damage as evaluated by the FOX assay after the exposure of FA-enriched U937 cells to t-BHP/Fe<sup>2+</sup> radical system for 30 min. Glycerophospholipids in the adult brain account for  $\approx$  20-25% of the dry weight (Farooqui et al., 2000). Most PUFAs used by the brain for the synthesis of these glycerophospholipids are not made in the central nervous system (CNS), but are transported there from the gastrointestinal tract (Horrocks and Yeo, 1999), from the diet or produced in the liver. Free radicals target cellular components indiscriminately, including lipids, proteins and DNA. Hydroperoxides, produced from these reactions can further decompose over time to other toxic substances such as malonyldialdehyde, which can damage adjacent cells, membrane-bound enzymes and receptors, resulting in membrane breakdown, cytotoxicity and enzyme modification (Ernster, 1993). It is, therefore, not surprising that there is growing evidence in support of free radical mediated damage in schizophrenia (Reddy and Yao, 1996), a disease also with an impaired antioxidant defense mechanism (Reddy et al., 1991).

#### 4.4.2 Antioxidants

The effectiveness of an antioxidant has been attributed partly to the intrinsic chemistry of the compound and partly to the environment in which that chemistry is expressed. The lipid-soluble antioxidants used in this study were added to CM as solutions in organic solvents; ethanol for  $\alpha$ -tocopherol and chloroform for the carotenoids. Since chloroform is immiscible with water, the solvent problem was circumvented by sonicating the carotenoid - supplemented CM, briefly in an ice bath, into small micelles prior to U937 cell addition, aiding cellular uptake. Carotenoids are unstable and the stock solutions may change over time due to oxidative breakdown or the carotenoid may come out of the solution due to its tendency to crystallize when stored at  $-20^{\circ}\text{C}$  in the freezer (Hess et al., 1991). To overcome this problem, stock solutions were stored only for 2 months at  $-20^{\circ}\text{C}$ .

Populations with low intakes of dietary antioxidants such as vitamins C, E and  $\beta$ -carotene appear to have a higher risk of early development of degenerative diseases commonly associated with aging (Gey, 1993; Heliovaara et al., 1994). A number of human, animal and *in vitro* studies have demonstrated the protective effects of  $\beta$ -carotene against lipid peroxidative damage (Kunert and Tappel, 1983; Franke et al., 1994). Supplementation of rats with 1 g/kg body weight of astaxanthin reduced the lysis of red blood cells subjected to oxidative stress (Miki, 1991). Also dietary supplementation with  $\beta$ -carotene has been shown to protect mice against lipid peroxidation induced by methyl mercuric chloride (Anderson and Anderson, 1993) and to reduce the concentration of biomarkers of lipid peroxidation detected in serum (Mobarhan et al., 1990; Gottlieb et al., 1993) and pentane in exhaled breath (Allard et al., 1994) in human subjects.

Ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin have received considerable attention as putative antioxidants with the ability to attenuate oxidative stress, ameliorating cellular dysfunction resulting from free-radical induced oxidative injury to cellular components. Biological antioxidants can be grouped into two groups: preventive – and chain – breaking antioxidants. Preventive antioxidants reduce the initiation of lipid peroxidation by suppressing the production of chain-initiating radicals whilst the other group interferes with propagative oxidative processes by trapping the chain –initiators and/or chain propagators (such as the alkoxyl and peroxy radicals) of peroxidation (Niki, 1987). Carotenoids are generally classified as preventive antioxidants due to their ability to deactivate singlet oxygen responsible for the formation of lipid hydroperoxides (Foote and Denny, 1968), thus suppressing hydroperoxide-dependent lipid peroxidation (Terao et al., 1980; Matsushita and Terao, 1980). Also, carotenoids are able to act as chain-breaking antioxidants (Krinsky and Deneke, 1982).

$\alpha$ -tocopherol is a chain-breaking antioxidant which acts by trapping peroxy radicals and in turn donating a hydrogen atom. On the other hand  $\beta$ -carotene or related carotenoids, seems to act by a mechanism in which the chain-propagating peroxy radical is trapped by the addition to the conjugated polyene system of the carotenoid other than by hydrogen donation (Burton and Ingold, 1984). The resulting carbon-centered radical is stabilised by resonance because of the delocalisation of the unpaired electron in the conjugated carotenoid polyene-system leading to chain termination. Terao (1989) reported that astaxanthin was more resistant to oxidative chain reaction than  $\beta$ -carotene, confirming the results obtained in this study (Fig. 4.13). At physiological concentrations, carotenoid contents of the human plasma are much higher than that found in some primates and non-primate mammals (Cutler, 1984). It has been

reported that the major carotenoid species of the human plasma include  $\beta$ -carotene at 0.1-0.2  $\mu\text{g/ml}$  and lycopene at 0.2-0.5  $\mu\text{g/ml}$  (Terao, 1989), concentrations much lower than those employed in this study.

Ascorbic acid (vitamin C) acts as a chain-breaking antioxidant (Niki et al., 1984). Although vitamin C is a potent water-soluble antioxidant, at high concentrations it is also known to act as a pro-oxidant, especially in the presence of iron (Carr and Frei, 1999). In this study however, its presence in U937 cells inhibited lipid peroxidation, when the cells were exposed to oxidation mixture for 30 min in HBSS at 37°C and 5%  $\text{CO}_2$  in a humid environment. Vitamin E (also known as  $\alpha$ -tocopherol) is more effective against lipid peroxidation than vitamin C (Niki et al., 1984). Vitamin E is the major chain-breaking antioxidant found in biological membranes (Burton et al., 1983; Ingold et al., 1987). This property has been reported to depend primarily on the phenolic group in its chromanol ring, rather than the side-chain (Burton and Ingold 1989; Niki et al., 1995b).

Humans accumulate both groups of carotenoids in the plasma and tissues. The distribution of each carotenoid is rather different among organs. For instance, zeaxanthin (ZX) and lutein were the major carotenoids in the macular area of the human retina, while  $\alpha$ ,  $\beta$ -carotene, lycopene, cryptoxanthin, zeaxanthin (ZX) and lutein are dominant in the human plasma (Palace et al., 1999). The major storage organs for carotenoids are the liver and adipose tissues (Parker, 1989). Dietary intake varies between individuals in serum concentrations (Parker 1989). Carotenoid concentrations vary between sexes, geographical location, age, alcohol consumption (Stahl and Sies, 1996) and cigarette smoking (Sinclair et al, 1990). Carotenoids show a sequence of astaxanthin >  $\beta$ -carotene, in terms of their ability to inhibit lipid peroxidation and this is



supported by previous studies (Terao, et al., 1992; Goto et al., 2001). This is in support of the results obtained in this study (Fig. 4.13).

Apart from a benign and transient yellowing of the skin in heavily supplemented individuals, no adverse effect of  $\beta$ -carotene has been reported (Diplock, 1995). Carotenoids are being increasingly investigated as important components of the diet, exerting powerful antioxidant influences on human health and diseases. For example, male and female sea-urchin gonads (rich in carotenoids) are a valuable food commodity in countries such as Japan and Portugal.

As well as their ability to quench singlet oxygen radicals, carotenoids can also react with free radicals. The quenching of singlet oxygen by carotenoids mainly leads to energy dissipation as heat whereas, the reactions of carotenoids or any of the other antioxidants with free radicals leads to electron transfer. However, the unpaired electron characterizing the free radical is not lost in these reactions, possibly leading to propagative peroxidative reactions in the absence of fresh antioxidants.

#### ***4.4.2.1 Combination of antioxidants***

$\alpha$ -tocopherol, being a fat-soluble antioxidant, is effective against oxidative injury in plasma membranes. However, it cannot prevent against damage in cytosolic regions. Therefore the results obtained with a mixture of vitamins C and E (Figs 4.14 to 4.16) did not come as a total surprise, although the highest antioxidant protection was expected with a mixture of vitamin C and either of the carotenoids particularly astaxanthin because the highest protection resulted from the use of astaxanthin on its own when compared to the other antioxidants used (Fig. 4.13). Adjunctive treatment of vitamins C and/or E in conjunction with antipsychotic drugs such as haloperidol and

clozapine are already in use in the treatment of schizophrenia (Aruoma and Halliwell, 1987; Linday et al., 1995). A combination of vitamin C and E has also been reported to possess a synergistic effect in inhibiting oxidative processes (Tappel, 1962; Leung et al., 1981; Cort, 1982).

The effects of combining antioxidants as a measure against lipid peroxidation have previously been investigated, in order to determine the co-operative interactions between these agents. This study demonstrates positive antioxidant interactions between  $\alpha$ -tocopherol and the carotenoids ( $\beta$ -carotene and astaxanthin) in agreement with previous studies by Palozza and Krinsky, 1991; 1992, reporting a delay in the loss of microsomal tocopherols in the presence of  $\beta$ -carotene.

It has been reported that dietary  $\beta$ -carotene can mediate an increase in the activities of the antioxidant enzymes: SOD, and CAT induced by high fat diets (Blakely et al., 1988). The concept that antioxidants interlock in their protective effects and that the levels of all the antioxidants in the network are important, rather than just a single antioxidant, raises the possible importance of supplementing with an antioxidant cocktail rather than a single antioxidant (Keaney and Frei, 1994). Polar carotenoids (the xanthophylls such as astaxanthin) are more effective in situations where free radical attack occurs at the lipid/aqueous interphase. However, if radicals were generated in the lipid phase, less polar carotenoids such as the carotenes will yield more antioxidant effect (Ojima et al., 1993).

The interaction of carotenoids and vitamin A with vitamin E is not as widely considered as the interaction between vitamins C and E. Studies have shown that the antioxidant effects of vitamin A, carotenoids and vitamin E are interchangeable in contrast to the

relationship between vitamins C and E. For instance, there is no strong evidence suggesting that vitamin A and/or carotenoids regenerate vitamin E from its radical form, whereas vitamin C does (Tesoriere et al., 1993). However, when these different antioxidants are present together, there is an additive effect for conferring resistance to lipid peroxidation (Packer, 1993). This effect could be due to the fact that the different antioxidants protect at different physical locations within the cell membranes rather than physical interactions between antioxidants. Vitamin E for example, mitigates oxidation at the outer surface while carotenoids and vitamin A are protective in the interior of the membrane (Niki et al., 1995a).

Furthermore,  $\alpha$ -tocopherol prevents the auto-oxidation of carotenoids at physiological concentrations (Handelman et al., 1991) and can scavenge the carotenoid peroxy radical before it propagates peroxidation (Niki et al., 1995a). Conversely, protection of vitamin E by carotenoids may also be important when singlet oxygen attack is a factor (Ojima et al 1993). Co-operative interactions between the antioxidant enzymes inherent in U937 cells, water- and fat-soluble dietary antioxidants, has been exploited in this research as potential treatments for schizophrenia.

#### **4.4.3 Conclusion**

The data in this study indicate that carotenoids are potent antioxidants in U937 cells after 30 min of induced oxidation with the oxidant mixture. Taken together, this report demonstrates that t-BHP/ $\text{Fe}^{2+}$  - induced oxidation in PUFA-enriched U937 cells is inhibited by the antioxidants: ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin, to varying degrees with astaxanthin being the most effective after 30 min of oxidation.

Compared to free radicals, lipid peroxidation products are generally more stable and capable of diffusing within cells or even escaping from the cell, thereby attacking targets far from the site of origin of the free-radical. Using the t-BHP: FeSO<sub>4</sub> system as a pro-oxidant, peroxidation was inhibited by an ascorbic acid concentration of 400 μM (Fig. 4.9), α-tocopherol at a concentration as low as 10 μM (Fig. 4.10), β-carotene (Fig. 4.11) and astaxanthin at 12 μM (Fig. 4.12).

Since oxidative stress places a burden on the entire cell/organ, it is not surprising that stress in one cell can affect the redox balance in other cells. The same principle applies to antioxidants. For example, *in vitro* experiments showed that vitamin C reduces the vitamin E radical (tocopheryl radical) back to vitamin E, thus extending the effectiveness of Vitamin E (Keaney and Frei, 1994; Halliwell and Gutteridge, 1999).

In conclusion, the present work demonstrates that the generation of lipid hydroperoxides increases with increasing polyunsaturated fatty acid concentrations, also depending on the degree of unsaturation. It also shows that lipid peroxidation by-products (lipid hydroperoxides) accumulate in U937 cells over time under oxidative stress. Indeed, pre-treating U937 cells with all the antioxidants (ascorbic acid, α-tocopherol, β-carotene and astaxanthin) used in this study showed potent inhibition of lipid peroxidation at certain concentrations, with astaxanthin exerting the highest antioxidant activity. A mixture of ascorbic acid and α-tocopherol gave the greatest antioxidant protection observed after 30 min of incubation with the oxidant mixture.

Overall, the data reported here provide more evidence in favour of the crucial role of the protective action of the dietary antioxidants against t-BHP/Fe<sup>2+</sup> induced lipid peroxidation with antioxidant combinations more potent than individuals.

## CHAPTER 5: LIPID PEROXIDATION EXPERIMENTS (PRELIMINARY STUDY BY HEADSPACE ANALYSIS)

### 5.1 INTRODUCTION

Free radical-induced lipid peroxidative damage in schizophrenia is being increasingly investigated using *in vitro* systems. The presence of membrane phospholipids at locations where these free radicals are generated render them accessible targets for lipid peroxidation since the polyunsaturated fatty acids (PUFAs) especially, are highly susceptible to reactions with free radicals (Halliwell and Gutteridge, 1990b). Furthermore, the measurement of generated hydrocarbons is rapidly becoming a tool for the analysis of lipid peroxidation in biological systems.

Both *in vitro* (North et al., 1994) and *in vivo* (Kivits et al., 1981; Kovaleva et al., 1989) studies have utilized the measurement of volatile hydrocarbons, mainly ethane and pentane, as a validated method of measuring lipid peroxidative damage. In studies conducted by Kivits et al., (1981), the feeding of rats with various fatty acids demonstrated that the oxidation of n-3 PUFAs yielded increased ethane levels in exhaled breath while n-6 PUFAs produced pentane. Since ethane is less easily metabolized to carbon dioxide (CO<sub>2</sub>) than pentane, it is considered to be a better indicator of lipid peroxidation (Allerheilgen et al., 1987). However, it has been shown that ethane is produced in smaller quantities than pentane in humans (Wade and Van Rij, 1985). A major problem with pentane apart from its ease of degradation to CO<sub>2</sub> is that GC-MS techniques do not differentiate it from isoprene because they co-elute on many gas chromatograph columns. As such pentane is easily confused with isoprene peaks generating inaccurate determinations (Springfield and Levitt, 1994).

The volatile hydrocarbon test is appealing because it is a useful, non-invasive method of measuring lipid peroxidation, both *in vivo* and *in vitro* in the study of several pathological conditions such as schizophrenia, where lipid peroxidative damage has

been extensively implicated. There is considerable interest in the measurement of hydrocarbons *in vivo* especially in humans, as a measure of lipid peroxidation since exhaled breath can be easily collected and trapped in closed chambers for analysis. Therefore, the purpose of this study was to quantify volatile hydrocarbons generated by U937 cells previously enriched with equal concentrations of different fatty acids, having variable degrees of susceptibility to peroxidative attack, before exposure to t-BHP/Fe<sup>2+</sup> at a concentration already established to induce lipid peroxidation by the measurement of lipid hydroperoxides (Chapter four).

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Materials**

Fatty-acid-free bovine serum albumin (BSA), stearic acid (SA), oleic acid (OA), arachidonic acid (AA), 0.4% Trypan Blue dye solution, Hanks balanced salt solution (HBSS), RPMI 1640, foetal bovine serum (FBS), 200 mM L-glutamine, 50 mg/ml gentamicin solution, HPLC-grade ethanol, tert butyl hydroperoxide, and ferrous sulfate heptahydrate (FeSO<sub>4</sub> · 7H<sub>2</sub>O) were obtained from Sigma-Aldrich, UK. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were supplied by Nu-chek Prep. Inc., Denmark. Sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) filters were from Jones Chromatography Ltd., UK.

### **5.2.2 Glassware and plastic preparations**

All glassware used was washed in de-con and nano-pure water, autoclaved at 121°C for 30 min and dried in an oven before use. All other apparatus were sterile with all U937 cell treatments performed under a class II laminar flow hood.

### 5.2.3 Cell culture

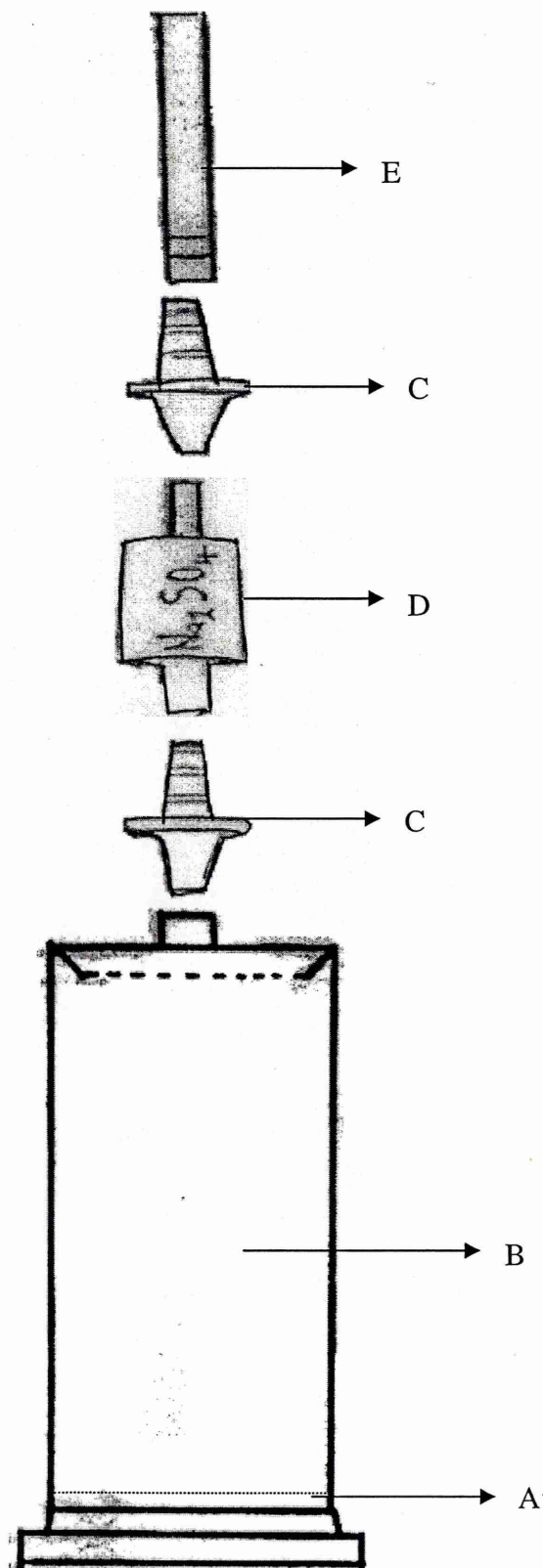
U937 cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine and 0.05 mg/ml gentamicin at 37°C in a humidified incubator with 5% CO<sub>2</sub>/95% air. For all experiments, cells were maintained at 0.5 x 10<sup>6</sup> cells/ml at viability > 90% by Trypan Blue dye exclusion method of viability measurements.

### 5.2.4 Cell treatments

Stock solutions of 100 mM fatty acid concentrations were prepared in ethanol and stored under nitrogen after sterile filtration using nylon syringe filters. By further dilutions, 10 mM stock solutions of all fatty acids were prepared. U937 cells adjusted to contain 0.5 x 10<sup>6</sup> cells/ml were treated with 10 mM aliquots of the FAs: SA, OA, AA, EPA, DHA or 0.4% v/v of ethanol (controls) complexed with aliquots of 4 mM stock solutions of fatty-acid-free BSA in a 2.5: 1 molar ratio. Cells were incubated in 20 ml suspensions in 75 cm<sup>2</sup> culture flasks at 37°C and 5% CO<sub>2</sub> in a humid environment. The final concentration of fatty acids in 20 ml cell suspensions per flask was 40 µM. After 24 h of incubation with the different FAs in freshly prepared CM, cells were washed twice, each time with 20 ml of HBSS/0.2% BSA by centrifugation at 250 g for 5 min at 4°C and re-suspended in 475 µl of HBSS in 50 ml centrifuge tubes. Stock solutions of 2 mM t-BHP and 5 mM FeSO<sub>4</sub> were freshly prepared in HBSS. To 475 µl of cell suspensions, 525 µl of t-BHP/Fe<sup>2+</sup> mixture was added to make a final volume of 1.0 ml and 750 µM t-BHP/Fe<sup>2+</sup> of oxidant concentration. Using sterile needles screwed to 20 ml sterile syringes (Becton Dickinson and Co., UK), cell suspensions were transferred into the syringes, with 20 ml headspace. Needles were carefully stuck into solid rubber bungs (size 15) ensuring that the needle barrel was completely inserted. The syringes were shaken and incubated at 37°C for 2 h. Thereafter, needles were carefully and

rapidly unscrewed and connected to automatic thermal desorption (ATD) tubes via  $\text{Na}_2\text{SO}_4$  filters (Fig. 6.1).





**Fig. 5.1** Schematic diagram of the procedure for the transfer of trapped gas produced by U937 cells. A: U937 cells treated with or without  $750 \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ ; B: 20 ml syringe; C: connector; D:  $\text{Na}_2\text{SO}_4$  filter; E: ATD tube.

The 20 ml headspace gas was trapped in Perkin-Elmer N930-7000 ATD tubes packed with Carbotrap 300 and capped. The headspace samples were analysed for ethane, butane and pentane using the GC-mass spectrometer (Perkin-Elmer Turbo Mass) (HPRF patent). ATD tubes were desorbed on to the cold trap for 20 min at 320°C and the volatile hydrocarbons liberated were swept onto 30m x 0.32 mm PLOT GC columns (PEQ) using helium (2 ml/min) as the carrier gas. The detection of hydrocarbons was performed by flame ionization with the oven set to 45°C for 3 – 10 min and ramped at 14°C per minute to 200°C, at which point it was held for 2 minutes.

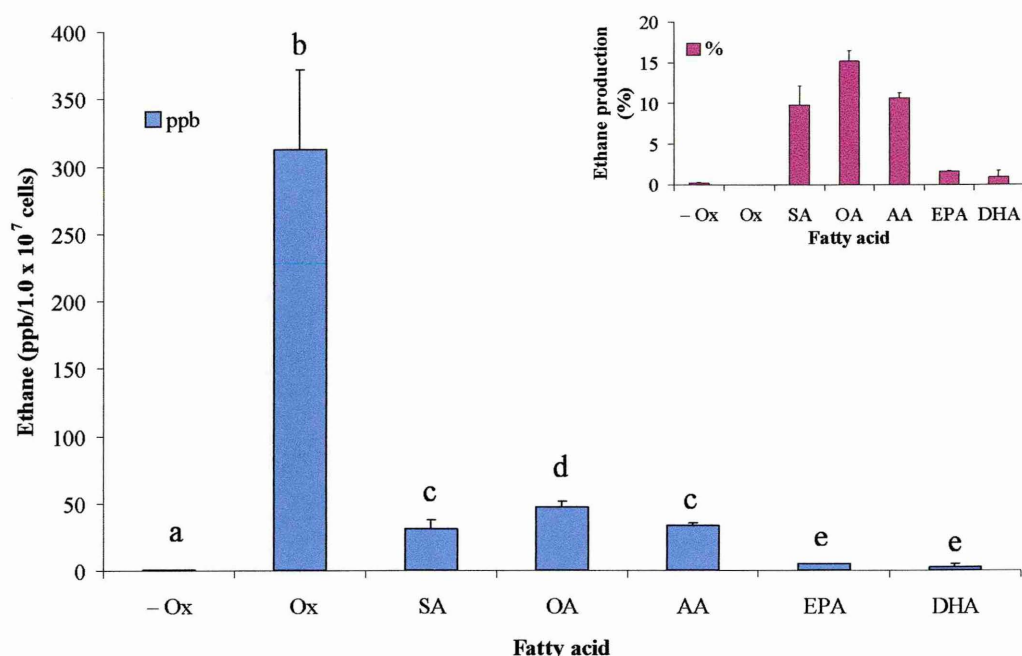
Quantification of ethane, butane and pentane production was accomplished by mass spectra (ethane at  $m/z = 30$ ; butane and pentane at  $m/z = 43$ ) and separation of authentic gas mixtures C<sub>1</sub>-C<sub>6</sub> hydrocarbons (Supelco, Inc.). Standard curves were established using C<sub>1</sub>-C<sub>6</sub> n-paraffins (methane, ethane, propane, n-butane and n-pentane at 15.0, 15.1, 14.9, 14.9 and 14.5 ppm, respectively) in helium, as the carrier gas, for the quantification of hydrocarbon peaks whilst a standard curve of ethane (0-1.5 ng/L) was run in nitrogen (N<sub>2</sub>), as the carrier gas, to quantify the standards. Ethane, butane and pentane eluted at 2.55, 9.62 and 12 minutes, respectively.

### 5.3 RESULTS

With increased lipid peroxidation in biological systems, the production of hydrocarbons, as by-products, can be used as a measure of the extent of such oxidative injury. Figs. 5.2 and 5.3 show the amounts of ethane and butane generated by U937 cells enriched with 40 µM levels of the fatty acids SA, OA, AA, EPA, DHA or 0.4% v/v, ethanol (for controls) for 24 h. This study began by determining whether t-BHP/Fe<sup>2+</sup> was capable of causing the generation of volatile hydrocarbons from fatty acid-treated U937 cells. To this end, the cells, pre-treated with 40 µM levels of SA,

OA, AA, EPA, DHA or 0.4% v/v, ethanol (for controls) were exposed to 750  $\mu\text{M}$  concentrations of t-BHP/ $\text{Fe}^{2+}$  for 2 h at 37°C. As shown in Fig. 5.2, treatment of U937 cells with t-BHP/ $\text{Fe}^{2+}$  significantly increased the production of ethane, a  $\text{C}_2$  hydrocarbon, most notably in control cells treated with the oxidant, but without any fatty acid supplementation (Ox). As expected, the lowest level of ethane was generated in control cells exposed neither to fatty acids nor oxidants (– Ox) but surprisingly, cell treatments with all FAs used especially the PUFAs (AA, EPA or DHA) appeared to inhibit ethane generation (Fig. 5.2).

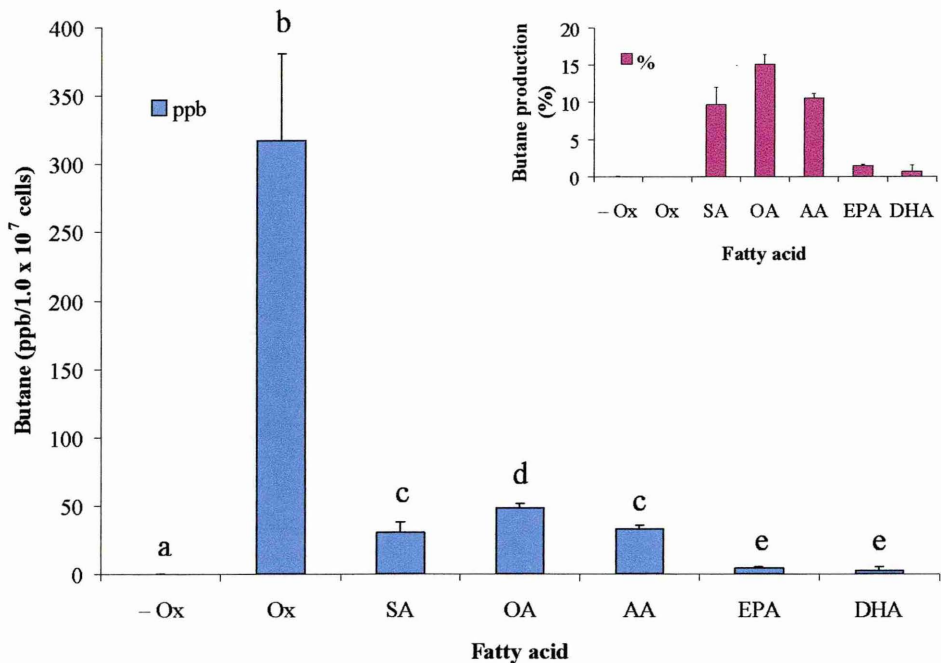
To compare results obtained in cells pre-incubated with equal concentrations of the various fatty acids used, ethane generation, in ppb/ $1.0 \times 10^7$  cells, was expressed as a percentage of that generated from oxidized controls (Ox), which represented 100%. A comparative study of unoxidized controls (– Ox), SA, OA, AA and EPA showed that the production of ethane was significantly lowest in controls incubated without oxidants (one-way ANOVA  $F = 237.65$ ,  $P < 0.01$ ,  $DF = 4$ ). The generation of ethane from U937 cells increased significantly from SA- to OA-, reducing significantly with AA- and EPA-pre-treatments. There was no significant difference between ethane generated from EPA- or DHA- treated cells ( $T = 1.39$ ,  $P > 0.05$ ,  $DF = 2$ ). Although EPA-treatment of U937 cells yielded ethane levels that were significantly higher than unoxidized controls (– Ox), there was no significant difference between DHA treatments and controls without oxidants ( $T = 1.29$ ,  $P > 0.05$ ,  $DF = 2$ ). Compared with un-supplemented U937 cells (– Ox), there was approximately 60%, 93%, 65%, 9% and 4% increases in ethane production with SA (18: 0), OA (18: 1), AA (20: 4), EPA (20: 5) and DHA (22: 6) – treatments, respectively.



**Fig. 5.2** Effect of fatty acid modification on free radical – mediated ethane generation in U937 cells ( $1.0 \times 10^7$ ). Data points represent means  $\pm$  95% CIs ( $n = 3$ ). The letters: a, b, c, d and e denote significant differences between means at  $P < 0.05$ .

The treatment of U937 cell controls with  $750 \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  (Ox) resulted in the generation of butane, a  $\text{C}_4$  hydrocarbon, as well (Fig. 5.3). Similar to the trend observed in U937 cells with ethane production, there was a striking difference between oxidant-treated controls (Ox) and all other treatments. U937 cell-treatments with  $40 \mu\text{M}$  SA generated butane levels significantly higher than oxidant untreated controls ( $T = 12.58$ ,  $P < 0.05$ ,  $\text{DF} = 2$ ). Whilst there was no significant difference in butane generation between unoxidized controls ( $- \text{Ox}$ ) and DHA – pre-treated U937 cells ( $T = 1.54$ ,  $P > 0.05$ ,  $\text{DF} = 2$ ), EPA – pre-treatment ( $T = 20.47$ ,  $P < 0.01$ ,  $\text{DF} = 4$ ) resulted in levels significantly higher than unoxidized controls ( $- \text{Ox}$ ). Tukey’s pairwise comparisons of butane generation from all FA-treated cells showed a significant increase in butane production from SA to OA (one-way ANOVA  $F = 98.06$ ,  $P < 0.01$ ,  $\text{DF} = 4$ ). However,

there was a marked decrease in butane levels in a gradual manner with corresponding cells pre-cultured with 40  $\mu$ M AA > 40  $\mu$ M EPA > 40  $\mu$ M DHA.

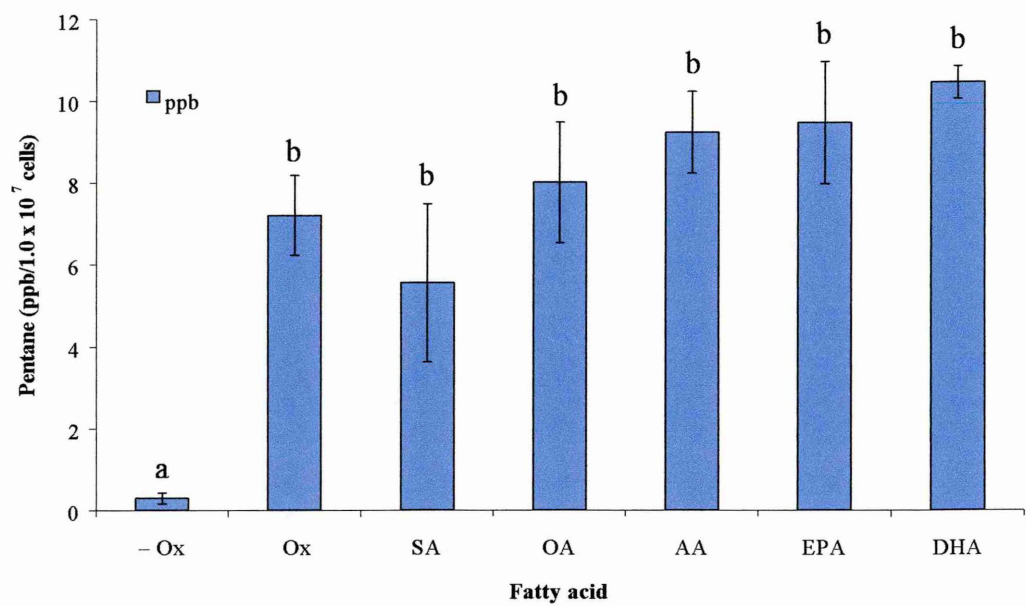


**Fig. 5.3** Oxidant-induced generation of butane by U937 cells grown for 24 h in FA-supplemented CM. Values of butane are expressed in ppb after 2 h of oxidative stress at 37°C. Means  $\pm$  95% CIs (n = 3). Letters: a – e indicate significant difference between treatments ( $P < 0.01$ ).

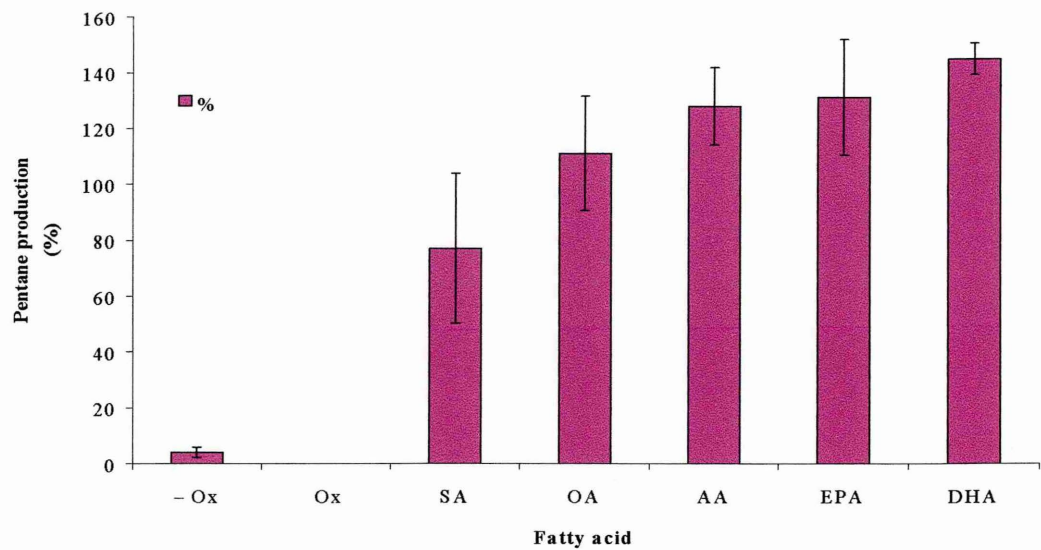
The suppression of volatile hydrocarbon production (especially ethane) from U937 cells enriched with FAs especially the PUFAs (EPA and DHA), observed in the present study, probably reflects the unsuitability of headspace analysis in the detection of the susceptibility of n –3 PUFAs to peroxidative damage, the abundance of which has previously been shown to increase the generation of ethane upon oxidative injury in biological systems.

Fig. 5.4 shows the amount of pentane generated by U937 cells enriched for 24 h with 40  $\mu$ M concentrations of the different fatty acids (SA, OA, AA, EPA or DHA).

Surprisingly, with induced oxidation, the production of pentane, a C<sub>5</sub> hydrocarbon, appeared to increase with increasing degree of FA unsaturation.



(a)



(b)

**Fig. 5.4** Effect of FA supplementation of U937 cell culture medium for 24 h on oxidant-induced generation of pentane. Values were expressed in ppb (a) and as percentages of that generated from oxidant treated controls (b). Data points represent means  $\pm$  95% CIs (n = 3). Letters a and b indicate significant differences between treatments at P < 0.01.

Pentane levels were significantly lower in unoxidized controls (– Ox) than in all other treatments involving induced oxidation (one-way ANOVA  $F = 58.07$ ,  $P < 0.01$ ,  $DF = 6$ ). Tukey's pairwise comparisons showed that there were no significant differences between oxidant-treated controls (Ox) and all FA-treatments. However, there were significant differences between the different FA-treatments. U937 cells treated with 40  $\mu$ M SA generated pentane levels significantly lower than all PUFA (AA, EPA or DHA)-treated cells. Contrary to expectations, there were no significant differences between OA, AA, EPA and DHA-pretreatments. Since pentane is an alkane produced by the decomposition of lipid peroxides generated from n-6 fatty acids, the highest levels were expected to be generated from AA-pre-incubated cells but this was not the case. However, it remains obvious that oxidative stress increases the generation of pentane.

From the results shown above, it is impossible to determine the origin of pentane generation from U937 cells pre-treated with the n-3 PUFAs without the inclusion of experiments determining the change in membrane fatty acid profile.

#### **5.4 DISCUSSION AND CONCLUSION**

The aim of this preliminary study was to investigate the production of volatile hydrocarbons from U937 cells grown in CM supplemented with/without different fatty acids, under conditions of oxidative stress. Lipid peroxidative damage to biological cell membranes especially when enriched with highly unsaturated fatty acids such as AA, EPA and DHA, is frequently associated with the generation of volatile hydrocarbons such as ethane and pentane. The present study demonstrated that the human lymphoma U937 cells generate butane as well as ethane and pentane during peroxidative processes with or without fatty acid modifications of the cellular phospholipids.

The presence of PUFAs of the n-3 series (DHA) was previously shown by North et al. (1994) to enhance the susceptibility of U937 cells to generate ethane in comparison to cells enriched with equal concentrations of oleic acid. In contrast, this preliminary study demonstrates that after 2 h of induced oxidative stress with 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  in U937 cells modified with different fatty acids (SA, OA, AA, EPA or DHA), the lowest amount of ethane was observed in cells pre-incubated with EPA (20: 5, n -3) or DHA (22: 6, n-3). The present study suggests that the treatment of U937 cells with the n-3 PUFAs (EPA or DHA) for 24 h, suppresses ethane production in comparison to SA, OA or AA. Collectively, pre-incubation of these cells with 40  $\mu\text{M}$  concentrations of all the fatty acids employed (SA, OA, AA, EPA and DHA), appeared to reduce the amount of ethane and butane generated upon oxidation, and increase pentane, when compared to oxidized controls (Ox). In contrast to findings from previous studies using neoplastic murine leukemia L1210 cells (Burns and Wagner, 1991), this study suggests that the treatment of U937 cells with AA, EPA or DHA conferred protective roles against oxidant-induced peroxidative damage as measured by ethane and butane generation in the following order: AA < EPA < DHA. Surprisingly, pentane was generated in the order: AA < EPA < DHA. It is important to note that pentane production (in ppb) by U937 cells in culture are much lower than ethane or butane levels. This could indicate that the n-3 lipid peroxides degrade to form ethane at a faster rate than the n-6's to pentane. Another possible explanation for the detection of lower pentane levels with n-6 pre-treated cells may be that most of the pentane generated was metabolized to carbon dioxide in the 2 h of inducing oxidative stress. A time course experiment of headspace sample collection is required to confirm this speculation.

Previously, studies *in vivo* of expired air from rats demonstrated a linear increase in ethane levels over a period of three hours when these rats were fed with n-3 rich diets



(cod liver oil), whereas there was no increase observed in ethane generation in control rats fed with n-3 poor diets (Odeleye et al., 1990). In contrast, the present data indicate that, in U937 cells, ethane generation is lower in EPA or DHA enriched cells post oxidation in comparison to oxidized controls. This could be because oxidation was induced for 2 h only and PUFA-treated cells require more time for the production of ethane in comparison to FA-untreated but oxidized controls (Ox). Or perhaps these cells need to be cultured in n-3 PUFA-rich medium for periods much longer than 24 h prior to oxidation experiments in order to determine susceptibility to peroxidative damage by ethane and pentane measurements. Since this is a preliminary study, more work is required to investigate ethane, butane and pentane generation in U937 cells cultured in PUFA-rich medium.

#### 5.4.1 Conclusion

The present study shows a relationship between lipid peroxidation and the generation of the volatile hydrocarbons, ethane (C<sub>2</sub>), butane (C<sub>4</sub>), and pentane (C<sub>5</sub>), with the C<sub>2</sub> and C<sub>4</sub> hydrocarbons both produced in like manners in U937 cell model systems. This suggests that ethane, pentane as well as butane can be formed as by-products of lipid peroxidation. The differential effects of the PUFAs in the generation of volatile hydrocarbons: ethane, butane and pentane, requires further investigation. With PUFA (AA, EPA or DHA) treatments, the present study demonstrates that these cells suppress the generation of ethane and butane when compared to oxidized controls (Ox) and at the same time increase pentane levels. Although the technique of GC headspace is rapid, sensitive and suitable for biological materials, involving no sample workup, whether the method is ideal for the measurement of peroxidative susceptibility as affected by the fatty acid composition of cellular phospholipids in biological samples *in vitro*, requires further study.

## CHAPTER 6: OXIDANT-INDUCED ARACHIDONIC ACID RELEASE

### 6.1 INTRODUCTION

Arachidonic acid (AA) is an important membrane component, required for the maintenance of cellular viability under normal and pathological conditions. The vast majority of the available arachidonic acid is stored in acylated form within membrane phospholipids, and the free acid is preferentially released from phospholipid molecules via the hydrolytic activity of form of the cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) enzyme (Tran et al., 1996). AA is a key intermediate in a diversity of signal transduction pathways. The rate limiting step in the initiation of the arachidonic acid cascade is its release from membrane phospholipids, resulting in the production of inflammatory mediators such as prostaglandins, leukotrienes and thromboxanes (Smith 1989; Rzigalinski et al., 1996). It has also been suggested that AA and/or its metabolites are involved in the regulation of intracellular free calcium levels (Mochizuki-Oda et al., 1993; Tornquist et al., 1994; Wu et al., 1994; Chow and Jondal, 1990) and the activation of low molecular weight GTPase activating proteins (Ligeti et al., 1993; Chuang et al., 1993; Homayoun and Stacey, 1993).

Agonist stimulated fatty acid mobilization by the calcium ionophore, A23187, is specific for AA and a few structurally related PUFAs such as EPA, which gives a quantitatively similar response to that of AA. DHA, however, is not released in response to agonists such as A23187 (Rosenthal et al., 1995). Studies with a variety of radiolabelled-PUFAs, show that agonist stimulated response is highly selective for fatty acids with a cis-5 double bond, but not particularly stringent for chain lengths over the range of C18 – C21 (Rosenthal et al., 1989). However, physiological conditions causing cell damage can result in the release of other fatty acids such as oleic acid and DHA (Rosenthal and Jones, 1988).

AA is an intermediate in the reacylation and deacylation of membrane phospholipids (Lands pathway). In this pathway, AA is cleaved from phospholipids by PLA<sub>2</sub> and re-incorporated by acyltransferases (MacDonald and Sprecher, 1989; Yamashita et al., 1997; Lands, 2000). In resting cells, reacylation prevails whilst deacylation is the dominant reaction in activated cells. AA incorporation into phospholipids is critically dependent on the availability of lysophospholipid receptors.

The human histiocytic lymphoma cell line (U937), is a rich source of cPLA<sub>2</sub>, and has been a standard line for the purification of the human form of the enzyme (Diez and Mong, 1990; Clark et al., 1990; Kramer et al., 1991). Therefore, in this study, this cell line was chosen to investigate oxidant-stimulated AA release and its inhibition by the incorporation of various known antioxidants. U937 cells contain high levels of the 110 kDa, Ca<sup>2+</sup>-dependent PLA<sub>2</sub> enzyme (Clark et al., 1990; Kramer et al., 1991; Sharp et al., 1991; Dennis 1994). PLA<sub>2</sub> has been implicated in schizophrenia (Gattaz et al., 1987; 1990). While several different types of PLA<sub>2</sub>s including the Ca<sup>2+</sup>-dependent and independent cPLA<sub>2</sub> have been identified in U937 cells, Clark et al (1990) reported that there was no measurable secretory PLA<sub>2</sub> able to block the Ca<sup>2+</sup>-stimulated AA release in these cells suggesting that cPLA<sub>2</sub> was mainly responsible for this release. It has recently been shown that phosphorylation of cPLA<sub>2</sub> coupled with an influx of Ca<sup>2+</sup> alone are not sufficient to activate the enzyme to release free AA in U937 cells (Burke et al., 1997). The differentiation of U937 cells, however, enables cPLA<sub>2</sub> to function in a catalytic fashion. While undifferentiated cells have large amounts of phosphorylated cPLA<sub>2</sub>s and can be stimulated to induce a calcium influx, they are unable to release AA. Recently, U937 cells have been reported to express both group IV PLA<sub>2</sub>s (cPLA<sub>2</sub>) and the group VI PLA<sub>2</sub> (iPLA<sub>2</sub>) (Hsu et al., 2000). But the inhibition of iPLA<sub>2</sub> with bromoenol lactone suicide substrate, suppressed neither AA incorporation nor its release

in U937 cells stimulated with A23187 (Hsu et al., 2000). However, Balboa and Balsinde (2002), showed that hydrogen peroxide induced AA release in U937 cells by the activation of iPLA<sub>2</sub> independent of cPLA<sub>2</sub> activity.

The cytosolic form of PLA<sub>2</sub> (cPLA<sub>2</sub>) is localized in the cytoplasmic fraction of unstimulated cell homogenates and translocates to the membrane fraction in response to physiological (sub-micro to micro – molar) elevations of intracellular free calcium. cPLA<sub>2</sub> is activated by phosphorylation followed by the translocation of the phosphorylated enzyme to the membrane in a Ca<sup>2+</sup>-dependent manner (Clark et al., 1991). cPLA<sub>2</sub> has a high preference for arachidonic acid at the sn-2 position of the phospholipid molecule (Leslie et al., 1988; Clark et al., 1991) whereas iPLA<sub>2</sub> does not (Ackermann et al., 1995). cPLA<sub>2</sub> is enriched in brain cells (Gattaz et al., 1995) playing an important role in the regulation of the physico-chemical properties of the neuronal membranes thus influencing receptor function and signal transduction (Farooqui et al., 1992).

Blood cells (Balsinde et al., 1988), plasma (Gattaz et al., 1987), serum (Gattaz et al., 1990; Nojonen et al., 1993) and platelet membranes (Gattaz et al., 1995) have frequently been used to draw analogies to brain tissues regarding membrane abnormalities in neuropsychiatric disorders. In these reports, PLA<sub>2</sub> activity was increased. The mechanism for PLA<sub>2</sub>-mediated neural injury was proposed to be by glutamate receptor-mediated over-stimulation of PLA<sub>2</sub>, producing changes in membrane phospholipid composition, permeability and fluidity. These changes in membrane properties, allow enhanced calcium influx, leading to an uncontrolled sustained increase in cytosolic calcium levels, which may in turn be responsible for increased lipid breakdown, proteolysis and a disruption in the membrane structure (Farooqui and

Horrocks, 1991). These processes along with the accumulation of lipid peroxidation by-products, over extended periods, may be responsible for neurodegeneration.

The calcium dependent isoform of cPLA<sub>2</sub> has been implicated in dopaminergic signaling (Vial and Piomelli, 1995) whilst the calcium-independent PLA<sub>2</sub> performs a key role in cellular fatty acid incorporation into phospholipids (Balsinde et al., 1995). Chronic oxidative stress was reported to result in a specific increase in calcium-independent PLA<sub>2</sub> activity (Kuo et al., 1995). Ross et al., (1999) reported increased calcium-independent PLA<sub>2</sub> activity in the temporal cortex of schizophrenic patients, and this was related to oxidative stress coupled with a dysfunction in the fatty acid metabolism in schizophrenia. They also reported a decrease in calcium-dependent PLA<sub>2</sub> activity in the temporal cortex, in relation to an increase in dopaminergic activity (Ross et al., 1999). Ross et al (1997) used two different analytical methods (fluorometric and radiometric) to measure serum PLA<sub>2</sub> in schizophrenic patients. In that study, PLA<sub>2</sub> activity was increased in the serum of schizophrenic patients when analyzed with the fluorometric method, whereas the other method showed no significant difference between patients and control subjects. Further analysis showed that each method measured the activity of two different PLA<sub>2</sub> enzymes: the calcium – dependent (requiring 100 – 1000 Nm Ca<sup>2+</sup>) and independent enzymes, and that the latter was the over-active enzyme in the schizophrenic patients observed.

The specificity of phospholipase enzymes often limits the choice of phospholipids that can be employed in the assay. Ancillary concerns when working with phospholipase assays include the fact that all cells contain the basic repertoire of lipolytic enzymes required to maintain normal phospholipid metabolism. Since products may be produced by several pathways, radiolabelled fatty acids can be liberated from the sn-2 position of

the phospholipids by a PLA<sub>2</sub>; the combined activity of a PLA<sub>1</sub> and a lysophospholipase; or even by the activity of a PLC and a lipase. This ambiguity precludes one from readily determining the positional specificity of an enzyme in biological cells.

The radiometric method of detection is the most sensitive and most widely used PLA<sub>2</sub> assay, requiring the use of radiolabelled phospholipids or fatty acids (as in this case), which can be expensive. The assay employed in the present study follows hydrolysis by directly measuring the liberation of AA, one of the hydrolysis products. This assay is discontinuous requiring the separation of the radioactive substrate from the labelled products. These separations are quite time consuming and laborious but very effective and sensitive. The most commonly used separation method employs thin layer chromatography (TLC) after solvent extraction of the lipids. Since all the reactants and products extract into the organic phase, it is necessary to separate these compounds, prior to quantification by liquid scintillation spectroscopy. TLC has the advantage that reactants and products can both be identified and quantified. In addition, if all the compounds are equally extracted, any losses in extraction will equally affect all components thereby generating accurate results. Even if the absolute amounts of the components vary, the ratio of the component as well as the rate calculated from it remains unaffected. TLC is a separation technique based on the principle that the components of a mixture are made to separate as a mobile phase acts in conjunction with a solid (stationary) absorbent phase.

Despite the physiological significance of the PLA<sub>2</sub>s, both its measurement and the understanding of its mechanism of action have posed several problems. As such, numerous assays including titrametric, acidimetric, radiometric, spectrophotometric and fluorometric methods have been developed over the years. The radiometric method

(Katsumata et al., 1986) was used in this study due to its high sensitivity and reproducibility although it suffers the demerits of being discontinuous, time-consuming and expensive. In this study,  $^3\text{H}$ -labelled AA was incorporated into phospholipids and the release of  $^3\text{H}$ -AA into the extracellular medium on stimulation was quantified. The intensity of the radioactivity released was used as an indication of the activity of  $\text{PLA}_2$ . This radiolabelled AA-release method has previously been used extensively in the investigation of the involvement of  $\text{PLA}_2$  in several processes including phospholipid metabolism, host defense, signal transduction and apoptosis (Balsinde et al., 1994; Zhang et al., 1998; Ramanadham et al., 1999; Hsu et al., 2000; Balboa and Balsinde, 2002).

Since oxidative stress,  $\text{PLA}_2$  and the PUFAs play an important role in schizophrenia, this study was designed to investigate the effects of oxidative stress on AA release and  $\text{PLA}_2$  activity. The present study is designed to extend the understanding of the stimulatory role of oxidants with PUFA-supplementation and the inhibitory effects of certain dietary antioxidants in the regulation of radiolabelled – arachidonic acid release in U937 cells, as a measure of the activity of a form of the phospholipase  $\text{A}_2$  ( $\text{PLA}_2$ ) enzyme.

## **6.2 MATERIALS AND METHODS**

### **6.2.1 Materials**

Fatty-acid-free bovine serum albumin (BSA), stearic acid (SA), oleic acid (OA), arachidonic acid (AA), phosphatidyl choline, 0.4% Trypan Blue dye solution, Hanks balanced salt solution (HBSS), RPMI 1640, foetal bovine serum (FBS), 200 mM L-glutamine, 50 mg/ml gentamicin solution, ascorbic acid, vitamin E,  $\beta$ -carotene, astaxanthin, HPLC-grade chloroform, hexane, diethyl ether, methanol and ethanol,

glacial acetic acid, potassium chloride (KCl), Iodine crystals, butylated hydroxy-toluene (BHT), tert butyl hydroperoxide, ferrous sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) and sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) were obtained from Sigma-Aldrich, UK. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were supplied by Nu-chek Prep. Inc., Denmark. Optiphase Hisafe 2 liquid scintillation cocktail and [5, 6, 8, 9, 11, 12, 14, 15- $^3\text{H}$ ] free arachidonic acid ( $^3\text{H}$ -AA, 100 Ci/mmol) were purchased from Perkin-Elmer Life Sciences, UK. LK5D – Whatman TLC plates were from Fisher Scientific, UK.

### **6.2.2 Glassware and plastic preparations**

All glassware used was washed in de-con and nano-pure water, autoclaved at 121°C for 30 min and dried in an oven before use. All other apparatus used were sterile with all U937 cell treatments performed under a class II laminar flow hood. Solvent extractions and thin layer chromatographic analysis were done in the designated area in the radiation suite.

### **6.2.3 Cell culture**

U937 cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine and 0.05 mg/ml gentamicin at 37°C in a humidified incubator with 5%  $\text{CO}_2$ /95% air. For all experiments, cells were maintained at  $0.5 \times 10^6$  cells/ml at viability > 90% by Trypan Blue dye exclusion method of viability measurements.

### **6.2.4 Assay of $^3\text{H}$ -AA release**

#### **6.2.4.1 With oxidants**

U937 cells (0.1 ml in CM) were labelled with 0.1  $\mu\text{Ci/ml}$  of  $^3\text{H}$ -AA in 0.9 ml of CM for 24 h at  $0.5 \times 10^6$  cells/ml in 24-well culture plates. Thereafter, cells were washed twice



with 1 ml 0.2% BSA (w/v) in HBSS (HBSS/0.2% BSA) by centrifuging at 250 g for 5 min at 4°C each time and re-suspended in 0.1 ml of HBSS only. Cells were washed in BSA to ensure the removal of all unincorporated freely-available fatty acids. Stock solutions of 2 mM t-BHP and 5 mM FeSO<sub>4</sub> were freshly prepared just before use in HBSS. Oxidative stress was induced by the addition of 0.9 ml of oxidant mixture (t-BHP and FeSO<sub>4</sub>) in HBSS (or HBSS alone for controls) to 0.1 ml of cell suspension in fresh 24-well plates and incubated at 37°C and 5% CO<sub>2</sub> in a humid environment. For the time-dependent assay, cells were incubated with a final oxidant concentration of 750: 750 µM t-BHP: FeSO<sub>4</sub> (or HBSS only for controls) over 6 h under the conditions stated above. The blank (0 h) experiment involved cells incubated in HBSS only for 6 h. The dose-dependent assay was carried out with cells incubated with 0: 0 to 1000: 1000 µM final concentrations of oxidant mixture for 4 h. All experiments involved radiolabelling with 0.1 µCi/ml <sup>3</sup>H-AA for 24 h at 37°C and 5% CO<sub>2</sub> in a humid environment.

All reactions were terminated rapidly by centrifuging at 4000 g for 3 min at 4°C prior to solvent extraction of lipids and thin layer chromatographic (TLC) analysis.

## **6.2.5 Treatment of cells with fatty acids (FAs)**

### ***6.2.5.1 With increasing concentrations***

Fatty acids (SA, OA, AA, EPA or DHA) were complexed to fatty-acid-free BSA at a molar ratio of 2.5: 1 and supplemented with <sup>3</sup>H-AA/CM before addition to cell suspensions as follows: ethanolic FA solutions ranging from 2.5 to 10 mM were prepared from a 100 mM stock solution and stored under nitrogen after sterile filtration using nylon syringe filters. FA: BSA complexes were prepared by the addition of an equal volume of 2.5 – 10 mM of the FA complexed with 1 – 4 mM of BSA respectively

and further diluted with CM supplemented with  $^3\text{H-AA}$  ( $^3\text{H-AA/CM}$ ) to a final volume of 0.9 ml. FA-supplemented culture medium (0.9 ml) was then added to 0.1 ml of cell suspension in each well of a 24 – well plate. Controls involved cells incubated with 0.4% (v/v) ethanol and  $^3\text{H-AA}$  only, representing the final ethanol concentration in all FA treatments, previously shown to have no effect on cell viability as measured by LDH analysis (section 2.3.5.3). Each well contained  $0.5 \times 10^6$  cells/ml and  $0.1 \mu\text{Ci/ml}$   $^3\text{H-AA}$ . Cells were incubated for 24 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in a humid environment. Final fatty acid concentration ranged from 10 to  $40 \mu\text{M}$ .

#### ***6.2.5.2 With a single FA concentration***

Cells were also incubated with  $0.1 \mu\text{Ci/ml}$   $^3\text{H-AA}$  and  $10 \mu\text{M}$  of each of the fatty acids: SA, OA, AA, EPA or DHA or ethanol only (controls) complexed to BSA at a molar ratio 2.5: 1. Incubations were performed in 24-well plates at  $0.5 \times 10^6$  cells/ml for 24 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in a humid environment. In one set of experiments, cells were incubated in HBSS only for 4 h without oxidation, in the other cells were subjected to oxidative stress ( $750 \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ ). Controls involved cells incubated with 0.4% (v/v) of ethanol complexed with an equal volume of 1 mM BSA.

#### ***6.2.5.3 Prolonged supplementation of cells with FAs***

U937 cells were continuously cultured for 18 days in CM supplemented with  $10 \mu\text{M}$  levels (final concentration) of the FAs (SA, OA, AA, EPA or DHA) at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in a humid environment. For controls, cells were incubated with 0.4% (v/v) ethanol (final volume) complexed with 1 mM BSA (equal volumes). All FAs were complexed with BSA in a 2.5: 1 molar ratio. Cells were maintained in 8 ml cell suspensions at  $0.5 \times 10^6$  cells/ml in  $25 \text{ cm}^2$  culture flasks every 3 days. The incubation medium was totally renewed with freshly prepared CM supplemented with  $10 \mu\text{M}$  of

the FA complex every 3 days. On day 17, U937 cells were re-adjusted to contain  $0.5 \times 10^6$  cells/ml and re-suspended in 24-well plates for a further 24 h in freshly prepared CM supplemented with 10  $\mu$ M of the fatty acid and 0.1  $\mu$ Ci/ml  $^3$ H-AA. Final volume of cell suspension per well was 1.0 ml. After a total of 18 days, the cells were subjected to oxidative stress for 4 h as in section 6.2.7.

Cell viability and cell density was monitored every 3 days and maintained at  $0.5 \times 10^6$  cells/ml at a viability > 90% by the Trypan Blue dye-exclusion method. Excess cell suspensions were discarded and treated appropriately.

#### ***6.2.5.4 Cell-associated free fatty acids ( $^3$ H-AA)***

U937 cells were treated with 10  $\mu$ M FAs (SA, OA, AA, EPA or DHA) and 0.1  $\mu$ Ci/ml following the protocol used above. Cells were incubated in 1 ml suspensions in 24-well plates at  $0.5 \times 10^6$  cells/ml at 37°C and 5% CO<sub>2</sub> in a humid environment for 24 h. Cells were washed twice with HBSS/0.2% BSA and incubated with the oxidant mixture (750  $\mu$ M t-BHP/Fe<sup>2+</sup>) for 4 h (see section 6.2.7). Thereafter, the reaction was stopped by centrifugation (4000 g for 3 min at 4°C) and 0.9 ml of supernatant were removed and discarded. Cell pellets (0.1 ml) were dislodged from walls of 1.5 ml eppendorf tubes followed by extraction of cellular lipids (see section 6.2.8.2).

All experiments were designed to include a set of U937 cells treated with 0.1  $\mu$ Ci/ml  $^3$ H-AA only for the determination of the total incorporated radioactivity into cellular phospholipids. These sets of experiments were stopped after 24 h of incubation at 37°C and 5% CO<sub>2</sub> in a humid environment prior to  $^3$ H-AA release experiments.  $0.5 \times 10^6$  cells/ml from each well, were centrifuged at 250 g for 5 min and 4°C, washed twice in

HBSS/0.2% BSA. Cells were then re-suspended in 0.1 ml of HBSS and extracted following the procedure for the extraction of cellular lipids (see section 6.2.8.2).

## **6.2.6 Supplementation of U937 cell culture medium (CM) with ascorbic acid, $\alpha$ -tocopherol, $\beta$ -carotene and astaxanthin**

### ***6.2.6.1 With increasing antioxidant concentrations***

100 mM stock solutions of ascorbic acid in water,  $\alpha$ -tocopherol in ethanol,  $\beta$ -carotene and astaxanthin in chloroform were prepared. Ascorbic acid and  $\alpha$ -tocopherol were stored at 2 – 8°C, while  $\beta$ -carotene and astaxanthin at – 20°C. By serial dilution, 4 – 16 mM ascorbic acid, 10 – 40 mM  $\alpha$ -tocopherol and 3 – 12 mM  $\beta$ -carotene and astaxanthin were prepared. Culture medium (CM) was supplemented with aliquots of 4 – 16 mM ascorbic acid, 10 – 40 mM  $\alpha$ -tocopherol and 3 – 12 mM  $\beta$ -carotene or astaxanthin, all to a final volume of 0.8 ml. Antioxidant supplemented CM (0.8 ml), was added to each of a 24 – well plate containing 0.1 ml of cell suspension after appropriate treatments (in the case of the carotenoids 0.8 ml of carotenoid – supplemented CM was sonicated for 5 s in an ice-bath and sterile – filtered using nylon syringe filters). Each well contained  $0.5 \times 10^6$  cells/ml. Final concentrations of antioxidants varied from 0 – 400  $\mu$ M for ascorbic acid, 0 – 40  $\mu$ M for  $\alpha$ -tocopherol and 0 – 12  $\mu$ M for  $\beta$ -carotene or astaxanthin. U937 cells were then incubated with each antioxidant for 10 min before the addition of 0.1 ml of 0.1  $\mu$ Ci/ml  $^3$ H-AA/CM and a further incubation for 24 h at 37°C and 5% CO<sub>2</sub> in a humid environment. Oxidized and unoxidized controls involved cells incubated with and without 750  $\mu$ M t-BHP/Fe<sup>2+</sup>, respectively.

Cells were also incubated in antioxidant-supplemented CM (0.9 ml) at 37 °C and 5% CO<sub>2</sub> in a humid environment for 10 min prior to the addition of 0.1  $\mu$ Ci/ml  $^3$ H-AA and 10  $\mu$ M AA, EPA or DHA (0.1 ml). Final antioxidant concentrations used varied from

100 – 400  $\mu\text{M}$  ascorbic acid; 10 – 40  $\mu\text{M}$   $\alpha$ -tocopherol; 3 – 12  $\mu\text{M}$   $\beta$ -carotene or astaxanthin. Aliquots of 2.5 mM PUFAs (AA, EPA or DHA) were complexed with 1 mM BSA and then added to the cell suspensions. The final volume of cell suspension/well was 1.0 ml. Plates were then incubated for a further 24 h under the conditions stated above. Oxidized and unoxidized controls were cells incubated with 10  $\mu\text{M}$  PUFA (AA, EPA or DHA) only (without antioxidant) with and without oxidation with 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ , respectively.

#### ***6.2.6.2 With individual and mixtures of antioxidants***

Cells were incubated with the highest concentration of each of the antioxidants used above (400  $\mu\text{M}$  ascorbic acid, 40  $\mu\text{M}$   $\alpha$ -tocopherol, 12  $\mu\text{M}$   $\beta$ -carotene or astaxanthin). The individual experiment involved the incubation of cells with single or mixtures of antioxidants (for 10 min) and then with 10  $\mu\text{M}$  of the PUFAs AA, EPA or DHA and 0.1  $\mu\text{Ci/ml}$   $^3\text{H-AA}$  following the protocols described above and incubated for a further 24 h (using 24-well plates) at 37°C and 5%  $\text{CO}_2$  in a humid environment. Each well contained  $0.5 \times 10^6$  cells/ml.

The blank experiments were incubated neither with antioxidant, PUFA nor oxidant but with the radiolabel only. Oxidant-untreated controls involved U937 cells cultured without media supplementation with neither antioxidant(s) nor PUFA but with oxidant. Oxidized controls were cells cultured without antioxidant(s) but with 10  $\mu\text{M}$  PUFA and oxidation. All incubations were performed for 24 h before oxidative stress was induced. Final antioxidant concentrations used were 400  $\mu\text{M}$  ascorbic acid, 40  $\mu\text{M}$   $\alpha$ -tocopherol, 12  $\mu\text{M}$   $\beta$ -carotene and 12  $\mu\text{M}$  astaxanthin.

For these sets of experiments with antioxidants, cells were washed twice in HBSS/0.2% BSA. Oxidation reactions followed the protocol in section 6.2.7. Unoxidized controls were not subjected to oxidation whilst oxidized controls were. All experiments were radiolabelled with 0.1  $\mu\text{Ci/ml}$   $^3\text{H-AA}$  (final concentration), supplemented with CM.

### **6.2.7 Oxidant-induced $^3\text{H-AA}$ release assay**

For all experiments involving radiolabeling for 24 h, cells were washed twice in 1 ml of HBSS/0.2% BSA by centrifuging each time at 250 g for 5 min at 4°C and re-suspending in 0.1 ml of HBSS. Cell suspensions were then transferred from 1.5 ml eppendorf tubes into new and sterile 24-well plates. Thereafter, 900  $\mu\text{l}$  of oxidant mixture in HBSS (freshly prepared from 2 mM t-BHP and 5 mM  $\text{FeSO}_4$  stock solutions, just before use) were carefully mixed with cell suspensions (0.1 ml) and incubated at 37°C and 5%  $\text{CO}_2$  for 4 h in a humid environment. The final oxidant concentration used in each well was 750: 750  $\mu\text{M}$  of t-BHP:  $\text{FeSO}_4$  in HBSS henceforth referred to as 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  unless otherwise stated.

All reactions were terminated after 4 h unless stated otherwise, by centrifugation at 4000 g for 3 min at 4°C prior to lipid extraction and TLC of extracts.

### **6.2.8 Lipid extraction**

#### ***6.2.8.1 $^3\text{H-AA}$ release into the incubation medium***

After the subjection of cells to oxidative stress, an 800  $\mu\text{l}$  aliquot of supernatant was removed and transferred into glass Pyrex tubes containing 4 ml of ice-cold chloroform: methanol (2: 1, v/v) (Folch et al., 1957), and 1 ml of 0.88% KCl was added as 25% of the existing volume. The mixture was shaken and allowed to separate into two layers. An aliquot of the bottom organic layer (0.5 – 2.0 ml) was transferred into a clean vial

and evaporated to dryness under a gentle stream of nitrogen. Extracts were re-suspended in 30  $\mu$ l of 3.33 mM cold AA in chloroform: methanol (2: 1, v/v) with 0.01% BHT in air-tight vials and stored for a maximum of 72 h at  $-20^{\circ}\text{C}$  prior to TLC analysis of  $^3\text{H}$ -AA released.

#### **6.2.8.2 $^3\text{H}$ -AA incorporated into phospholipids or cell-associated**

For the analysis of incorporated  $^3\text{H}$ -AA or cell-associated free  $^3\text{H}$ -AA, 0.8 ml (8 volumes) of ice-cold, chloroform: methanol (2: 1, v/v) was added to 0.1 ml of cell suspension. Cells were sonicated for 3 x 10 s with an interval of 10 s in an ice bath followed by the rapid addition of 0.2 ml (0.2 volumes) of ice-cold methanol. Suspensions were mixed briefly with a vortex mixer and centrifuged at 4000 g for 3 min at  $4^{\circ}\text{C}$ . Centrifugation was used to convert all non-lipid residues into compact pellets instead of filtration due to the small sample size. 0.7 ml of supernatant was transferred into labelled Pyrex tubes and 0.2 ml (25% of the existing volume) of 0.88% KCl solution was added. Tubes were well shaken and allowed a few minutes for separation into two phases. Aliquots of 0.1-0.2 ml of the bottom layer were transferred into sterile vials and evaporated to dryness under a stream of nitrogen. Cellular extracts were re-suspended in 30  $\mu$ l of 3.33 mM unlabelled AA in chloroform: methanol (2: 1, v/v with 0.01% BHT) in air-tight vials and stored for a maximum of 72 h at  $-20^{\circ}\text{C}$  prior to TLC analysis of cell-associated  $^3\text{H}$ -AA. For  $^3\text{H}$ -AA incorporated into phospholipids, extracts were re-suspended in 30  $\mu$ l of chloroform: methanol (2: 1, v/v with 0.01% BHT) and stored in airtight vials at  $-20^{\circ}\text{C}$  before TLC.

#### **6.2.9 Thin layer chromatographic separations**

A solvent system of hexane: diethyl ether: acetic acid, 70: 30: 1.3 (v/v/v) was made up and stored in Schott bottles. Using a micro-Pasteur pipette, 10-20  $\mu$ l aliquots of lipid

extracts were spotted on LK5D plates under a stream of nitrogen gas with reference phosphatidyl choline and unesterified AA co-chromatographed as standards on separate lanes for identification purposes. The plates were run in a chamber pre-saturated with 100 ml of the solvent system above. After 50 – 55 min, plates were dried with cool air from a hair dryer for ~ 30 s in a fume cupboard until free of the acetic acid odour. Thereafter, plates were exposed to iodine vapour produced from iodine crystals in a closed tank. Lipid spots corresponding to standard phospholipid – (for total AA incorporation) and AA – (for  $^3\text{H}$ -AA release into the supernatant and cell-associated  $^3\text{H}$ -AA) bands were outlined with a needle. The TLC plates were removed from the tank and iodine was allowed to sublime. Spots ( $\leq 2 \text{ cm}^2$ ) were scraped with a scalpel and transferred via a small plastic funnel into 7 ml scintillation vials containing 0.4 ml of 1%  $\text{Na}_2\text{S}_2\text{O}_3$ , in order to reduce the iodine to iodide ion, thus decolorizing the mixture and minimizing quenching (as modified by Horrocks and Ansell, 1967). 4.0 ml of optiphase hisafe 2 liquid scintillation cocktail was added, mixed vigorously by vortexing and radioactivity was quantified by liquid scintillation spectroscopy in disintegrations per minute using the 1219 Rackbeta liquid scintillation counter by LKB Wallac.

Extracellular  $^3\text{H}$ -AA release was expressed as the percentage of radioactivity released into the culture medium compared with total incorporated radioactivity. Cell-associated, free  $^3\text{H}$ -AA was expressed as the percentage of radioactivity of free  $^3\text{H}$ -AA in cells compared with total incorporated radioactivity.

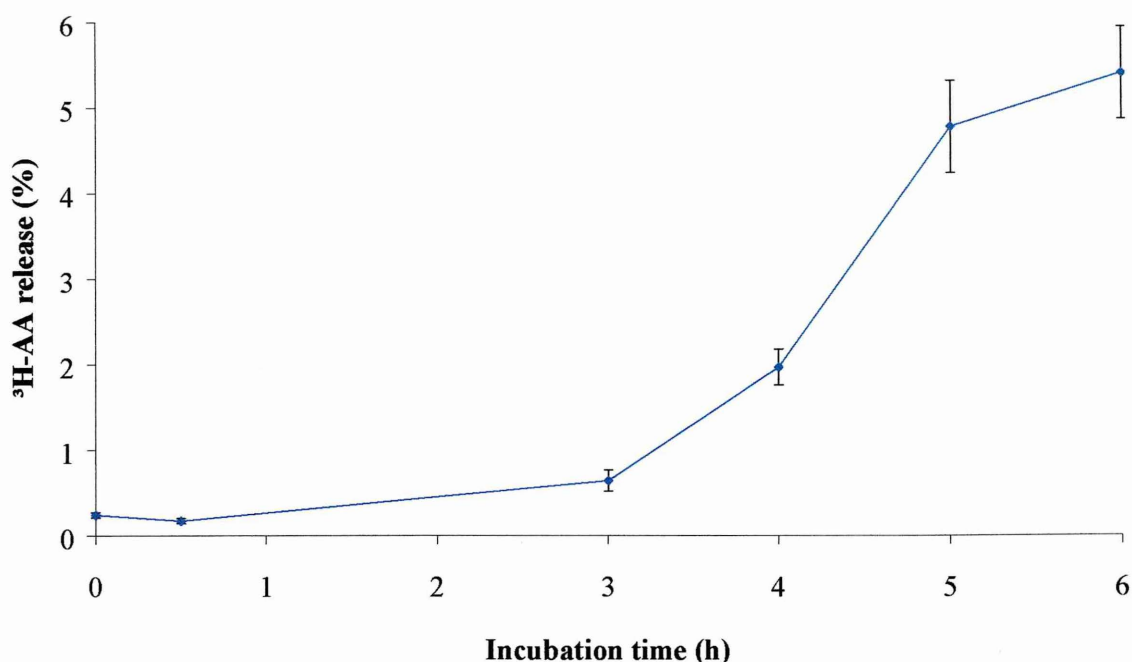
### 6.3 RESULTS

Initial experiments were performed to determine oxidant-stimulated release of [ $^3\text{H}$ ]-labelled arachidonic acid from undifferentiated U937 cells. In a previous chapter, a



mixture of t-BHP and FeSO<sub>4</sub> induced both dose- and time- dependent lipid peroxidative damage in U937 cells. In this study, the question of whether induced-oxidative stress was capable of causing the release of <sup>3</sup>H-AA into the extracellular fluid was investigated, since PLA<sub>2</sub> is present in high concentrations in U937 cells (Kramer et al., 1991). For this purpose, U937 cells were treated with or without varying amounts of the oxidant mixture and for various periods of time. It was found that <sup>3</sup>H-AA was released from prelabelled cells after 4 h of incubation at 37°C under the conditions tested as shown in (Fig. 6.1). The release was not significant between 0 and 3 h of incubation with 750 μM t-BHP/Fe<sup>2+</sup> but afterwards it proceeded linearly from 3 to 6 h therefore suggesting that oxidative stress enhances free AA release in U937 cells. The use of HBSS/0.2% BSA to wash the cells after 24 h of radiolabelling allows the trapping of all-free <sup>3</sup>H-AA remaining in the culture medium. BSA binds fatty acids, making it unavailable for cellular incorporation into phospholipids. Thus, washing of cells twice ensures the removal of unmetabolized AA, prior to the release experiments.

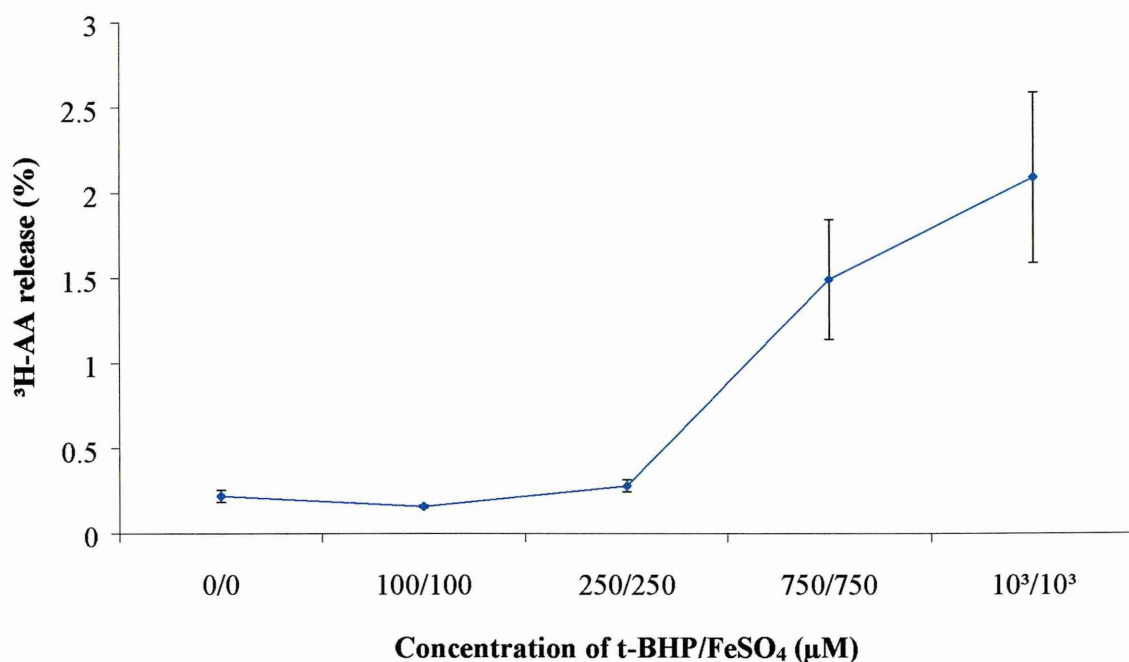
Fig. 6.1 shows the effects of treating U937 cells with 750 μM t-BHP/Fe<sup>2+</sup> in HBSS over a period of 6 h at 37°C and 5% CO<sub>2</sub> in a humid environment. Reactions were stopped at different time points, and free <sup>3</sup>H-AA in the extracellular medium was isolated by TLC and expressed as a fraction of the total incorporated fatty acid by liquid scintillation counting.



**Fig. 6.1** Time course of <sup>3</sup>H-AA release in U937 cells treated with the oxidant mixture of 750: 750  $\mu$ M of t-BHP: FeSO<sub>4</sub> (750  $\mu$ M t-BHP/Fe<sup>2+</sup>). Means  $\pm$  95% CIs of triplicate independent experiments (n = 4).

Since in previous studies 750  $\mu$ M t-BHP/Fe<sup>2+</sup> gave substantial detectable effects on both cell membrane integrity and lipid peroxidation, the effects of oxidative stress on <sup>3</sup>H-AA release was observed initially using that same concentration in U937 cells monitored over time. Increasing effects of t-BHP/Fe<sup>2+</sup> on <sup>3</sup>H-AA release were observed in cells after periods  $\geq$  3 h of incubation and maximal at 5 h, under the conditions stated above (One-way ANOVA  $F = 383.51$ ,  $P < 0.01$ ,  $DF = 5$ ). Tukey's comparisons showed no significant difference between the 0 and  $< 3$  h time periods. However, <sup>3</sup>H-AA release was significant after 3 h when compared to controls (0 h). <sup>3</sup>H-AA release increased over time in a linear fashion ( $R^2 = 0.955$ ,  $P < 0.01$ ). There was a time lag of about 3 h before t-BHP/Fe<sup>2+</sup>-induced AA release proceeded linearly, for 2 more hours but at a slower rate after 5 h. Based on these results, all subsequent experiments involved challenging U937 cells with t-BHP/Fe<sup>2+</sup> for 4 h prior to AA release analysis.

Thereafter, U937 cells labelled with 0.1  $\mu\text{Ci/ml}$  of  $^3\text{H-AA}$  were exposed to different concentrations of  $\text{t-BHP/Fe}^{2+}$  for 4 h (Fig. 6.2).



**Fig. 6.2** Concentration – dependent response of U937 cells to  $^3\text{H-AA}$  release with varying concentrations of  $\text{t-BHP/Fe}^{2+}$ . Means  $\pm$  CIs of two independent experiments ( $n = 4$ ).

Fig. 6.2 shows the dose-response of the  $\text{t-BHP/Fe}^{2+}$  effect on the release of  $^3\text{H-AA}$  from pre-labelled U937 cells. There was no significant increase in AA release at concentrations  $\leq 250 \mu\text{M}$  of the oxidant mixture compared with controls (One-way ANOVA  $F = 160.41$ ,  $P < 0.01$ ,  $DF = 4$ ). Again, maximal effects of the oxidant-induced AA release were observed at a concentration  $\geq 750 \mu\text{M}$  when compared to controls. As shown in Figs. 6.1 and 6.2,  $750 \mu\text{M}$   $\text{t-BHP/Fe}^{2+}$  induced a dose - and time - dependent release of  $^3\text{H-AA}$  in these cells.

### 6.3.1 With fatty acid concentrations

To examine the role of fatty acids on  $^3\text{H}$ -AA release, U937 cells were cultured in CM supplemented with varying concentrations of fatty acids (SA, OA, AA, EPA or DHA) as well as  $0.1\ \mu\text{Ci/ml}$  AA for 24 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in a humid environment. Afterwards, cells were challenged with  $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for 4 h in HBSS.

**Table 6.1** Effects of SA and OA (after 24 h) on oxidant-induced  $^3\text{H}$ -AA release. Values are expressed as a percentage of the total incorporated  $^3\text{H}$ -AA. Means  $\pm$  CIs (n = 4).

Concentration	SA	OA
Unoxidized control	$0.3018 \pm 0.1155$	$0.6775 \pm 0.0557$
Oxidized control	$2.0104 \pm 0.1150$	$1.6212 \pm 0.1039$
$10\ \mu\text{M}$	$2.0397 \pm 0.2744$	$1.5726 \pm 0.1019$
$20\ \mu\text{M}$	$1.9890 \pm 0.1665$	$1.5379 \pm 0.1330$
$30\ \mu\text{M}$	$2.3597 \pm 0.2962$	$1.5989 \pm 0.0526$
$40\ \mu\text{M}$	$2.4626 \pm 0.2213$	$1.6223 \pm 0.1228$

Pre-incubation of cells for 24 h with  $10 - 40\ \mu\text{M}$  concentrations of SA or OA (Table 6.1) showed no significant difference between  $^3\text{H}$ -AA released into the extracellular medium when compared to oxidized controls ( $0\ \mu\text{M}$ ). For SA (One-way ANOVA  $F = 89.78$ ,  $P < 0.01$ ,  $\text{DF} = 5$ ), Tukey's pairwise comparisons showed a significant difference between unoxidized controls and all treatments involving the oxidant. Similarly with OA (Kruskall-Wallis  $H = 12.28$ ,  $P < 0.05$ ,  $\text{DF} = 5$ ), there was a significant difference between unoxidized controls and all other treatments. Comparing all treatments with oxidants (oxidized controls to  $40\ \mu\text{M}$ ), there was no significant difference observed (Kruskall-Wallis  $H = 3.83$ ,  $P > 0.05$ ,  $\text{DF} = 4$ ). Neither the presence of SA nor OA in

concentrations varying from 0 – 40  $\mu\text{M}$  significantly affected the release of  $^3\text{H}$ -AA into the cell supernatants induced-oxidation in U937 cells when compared to oxidant-treated controls (Table 6.1).

**Table 6.2** Effects of AA, EPA or DHA (after 24 h) on the release of  $^3\text{H}$ -AA into the extracellular medium. Means  $\pm$  CIs (n = 4).

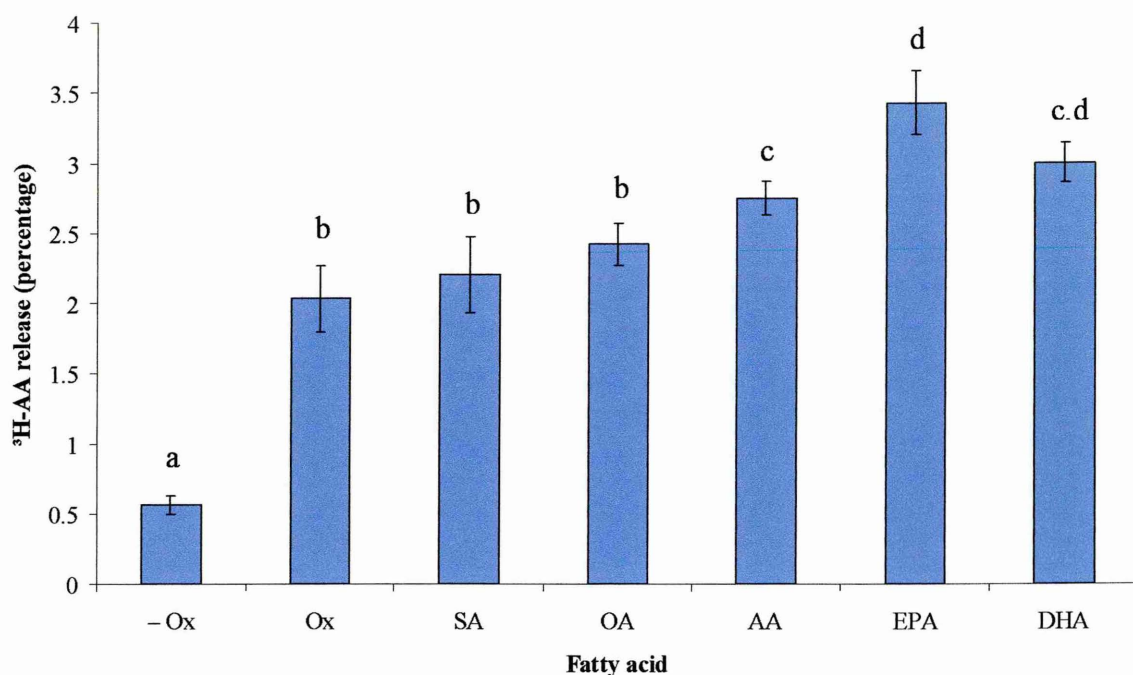
Concentration	AA	EPA	DHA
Unoxidized control	0.4094 $\pm$ 0.0562	0.4286 $\pm$ 0.0922	0.5257 $\pm$ 0.1699
Oxidized control	2.0146 $\pm$ 0.2528	2.2391 $\pm$ 0.1429	1.6136 $\pm$ 0.1911
10 $\mu\text{M}$	3.9517 $\pm$ 0.4920	5.0210 $\pm$ 0.9812	4.2215 $\pm$ 0.7375
20 $\mu\text{M}$	6.9470 $\pm$ 0.7720	7.8794 $\pm$ 0.7136	4.8876 $\pm$ 1.3070
30 $\mu\text{M}$	6.6159 $\pm$ 1.1044	7.6487 $\pm$ 1.5199	6.9101 $\pm$ 0.9959
40 $\mu\text{M}$	8.1778 $\pm$ 1.4553	8.4568 $\pm$ 0.8343	6.5364 $\pm$ 0.7934

To assess the effects of PUFAs on oxidant-induced AA release, cells were pre-treated with varying concentrations of each of the PUFAs (AA, EPA or DHA) and the radiolabel ( $^3\text{H}$ -AA) for 24 h (Table 6.2). Thereafter, cells were washed twice in HBSS/0.2% BSA to ensure the removal of unincorporated free fatty acids. Using the oxidant mixture at 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  concentration, cells were challenged for 4 h at 37°C and 5%  $\text{CO}_2$  in a humid environment. With AA-pretreated cells (Table 6.2),  $^3\text{H}$ -AA release proceeded linearly from oxidized controls to 20  $\mu\text{M}$  levels, after which it began to plateau (One-way ANOVA  $F = 115.70$ ,  $P < 0.01$ ,  $\text{DF} = 5$ ) (Table 6.2). Likewise, the same was observed with EPA-treatments of U937 cells (One-way ANOVA  $F = 117.49$ ,  $P < 0.01$ ,  $\text{DF} = 5$ ). DHA-pretreatments generated a linear increase in the release of  $^3\text{H}$ -AA over the concentration range of 0 – 30  $\mu\text{M}$  DHA levels (One-way ANOVA  $F = 69.20$ ,  $P < 0.01$ ,  $\text{DF} = 5$ ). Unlike results obtained from cells

pretreated with the saturated (SA) or the monounsaturated (OA) fatty acids, pre-incubation of U937 cells with the PUFAs (AA, EPA or DHA) produced a dose-dependent elevation in  $^3\text{H}$ -AA release into the extracellular medium as quantified by liquid scintillation spectroscopy following TLC separations. Oxidant treatment with the radical-mediated system of  $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  in serum free medium (HBSS) for 4 h indicated that the PUFAs (AA, EPA or DHA) generated a concentration-dependent increase in release of  $^3\text{H}$ -AA into the supernatant. Tables 6.1 and 6.2 suggest that  $^3\text{H}$ -AA release may be coupled to peroxidation in U937 cells. For all experiments with SA, OA, AA, EPA or DHA, there was a dramatic enhancement in U937 cell release of  $^3\text{H}$ -AA in oxidant-treated controls compared to those oxidant-untreated controls, with an average increase  $> 200\%$  to  $< 600\%$ .

The stimulation of PUFA-enriched U937 cells with the oxidant mixture resulted in increased AA release by 96% (AA), 124% (EPA) and 162% (DHA) in comparison to oxidized controls when used at  $10\ \mu\text{M}$  PUFA concentrations (Table 6.2). There was a linear relationship between PUFA concentration and oxidant-induced AA release: AA ( $R^2 = 0.936$ ,  $P < 0.01$ ); EPA ( $R^2 = 0.914$ ,  $P < 0.01$ ) and DHA ( $R^2 = 0.923$ ,  $P < 0.01$ ). Since a marked increase in  $^3\text{H}$ -AA release was observed with all three PUFA treatments at  $10\ \mu\text{M}$  levels when compared to oxidized controls, subsequent experiments involving AA, EPA or DHA were conducted at this concentration.

To provide further evidence for the coupling of oxidant-induced  $^3\text{H}$ -AA release to the vulnerability of U937 cells pretreated with the fatty acids SA, OA, AA, EPA or DHA, experiments were conducted with equivalent concentrations of each of the fatty acids ( $10\ \mu\text{M}$ ) and  $0.1\ \mu\text{Ci/ml}$   $^3\text{H}$ -AA for 24 h before subjection to the same level of oxidative stress ( $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  in HBSS) (Fig. 6.3) or HBSS only (Fig. 6.4).



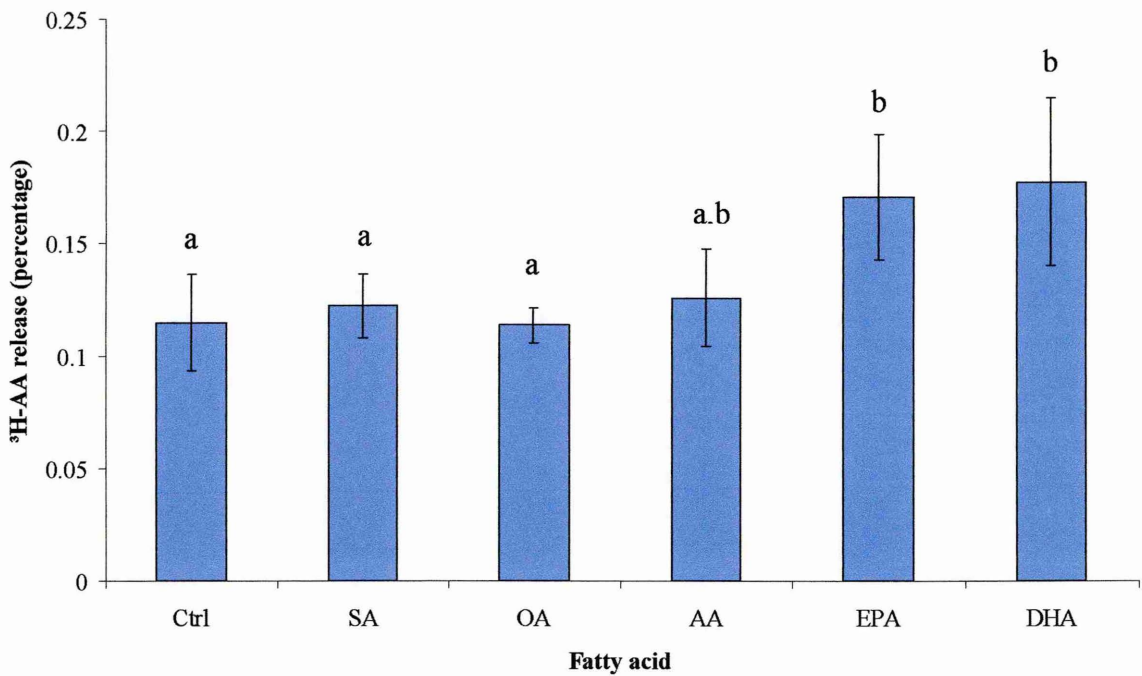
**Fig. 6.3** Effects of 24 h incubation with 10  $\mu$ M levels of SA, OA, AA, EPA or DHA and  $^3\text{H-AA}$  (0.1  $\mu\text{Ci/ml}$ ) on AA mobilization in U937 cells treated with 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ . Means  $\pm$  95% CIs of two independent experiments ( $n = 4$ ). Letters: a, b, c and d represent significant differences between treatments at  $P < 0.01$ .

Statistical analysis by one-way ANOVA ( $F = 131.25$ ,  $P < 0.01$ ,  $DF = 6$ ), showed no significant difference between oxidized controls and 10  $\mu\text{M}$  SA or OA, although there was an increase in  $^3\text{H-AA}$  release with increasing unsaturation (Fig. 6.3). There was a significant increase with PUFA treatments when compared to oxidant-treated controls. Comparative differences between cells treated with 10  $\mu\text{M}$  SA, OA, AA, EPA or DHA demonstrated that the presence of the PUFAs promoted increased  $^3\text{H-AA}$  hydrolytic release of 35% (AA), 69% (EPA) and 48% (DHA) of the total incorporated  $^3\text{H-AA}$  compared to controls. There was a marked increase ( $P < 0.0001$ ) in  $^3\text{H-AA}$  release in U937 cells exposed to oxidants compared to unoxidized controls ( $- \text{Ox}$ ). AA release into the extracellular medium was significantly higher in EPA- than AA- treated cells ( $P < 0.01$ ). However, there were no significant differences observed either between EPA-



and DHA-treatments or AA and DHA pre-treated cells. Surprisingly, a sequential trend in the stimulation of AA release with increasing degree of fatty acid unsaturation, was not observed. On the average, though not significant ( $P > 0.01$ ), EPA- but not DHA-treatment stimulated the highest AA release from U937 cells in this comparative experiment (Fig. 6.3).

Examination of the effects of incubating FA-treated U937 in HBSS only (Fig. 6.4) demonstrated enhanced AA release with EPA and DHA compared to controls even though there was no oxidation involved (One-way ANOVA  $F = 5.62$ ,  $P < 0.05$ ,  $DF = 5$ ).



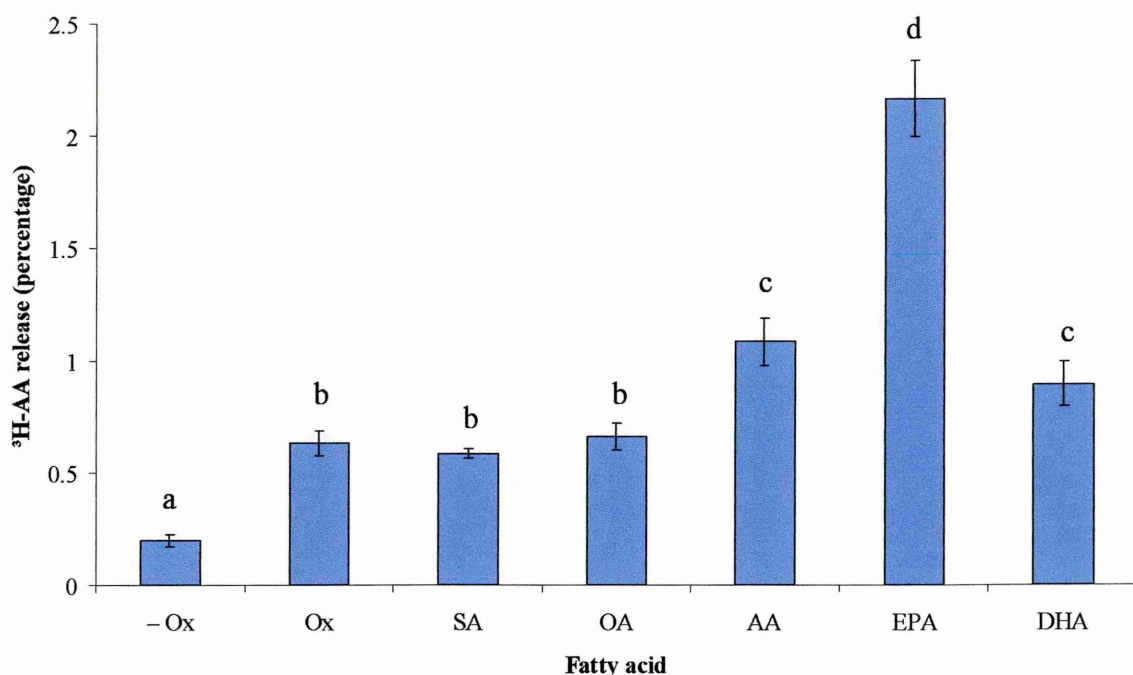
**Fig. 6.4** Effects of HBSS only on AA release from U937 cells pre-treated for 24 h with 10  $\mu\text{M}$  fatty acids and  $^3\text{H-AA}$ . \* Represents significant differences at  $P < 0.05$  (one-way ANOVA followed by Tukey's pairwise comparisons), between FA treatment and controls (ctrl). Means  $\pm$  95% CIs ( $n = 4$ ). Letters: a and b denote significant differences between means ( $P < 0.05$ ).



There was no significant difference between controls, SA-, OA- and AA-treated cells. AA release from EPA- and DHA-treated cells, were 48% and 54% higher than controls, respectively (Fig. 6.4).

Taken together, comparisons of results between FA-enriched U937 cells treated with and without oxidants (Fig 6.3 and Fig. 6.4, respectively), showed that there was a marked increase in AA release when the cells were stimulated with 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  than without. These results suggest that the presence of EPA or DHA on its own in U937 cells stimulates AA-release with or without oxidants when compared with controls, SA or OA. However, AA-treatment was neither significantly different from controls nor EPA/DHA when cells were incubated for 4 h without the oxidant (Fig. 6.4).

To investigate the possible involvement of prolonged fatty acid – pretreatment of U937 cells, oxidant-induced  $^3\text{H}$ -AA release after 18 days of CM-supplementation with 10  $\mu\text{M}$  concentrations of the fatty acids SA, OA, AA, EPA or DHA was determined. The culture medium was renewed every 3 days after viability measurements > 90% and re-adjustments of the cell density to  $0.5 \times 10^6$  cells/ml both monitored by the Trypan Blue dye-exclusion method.



**Fig. 6.5** Effects of prolonged fatty acid treatments (18 days) on AA release from U937 cells. Means  $\pm$  95% CIs (n = 4). Letters: a, b, c and d represent significant differences between means ( $P < 0.01$ ).

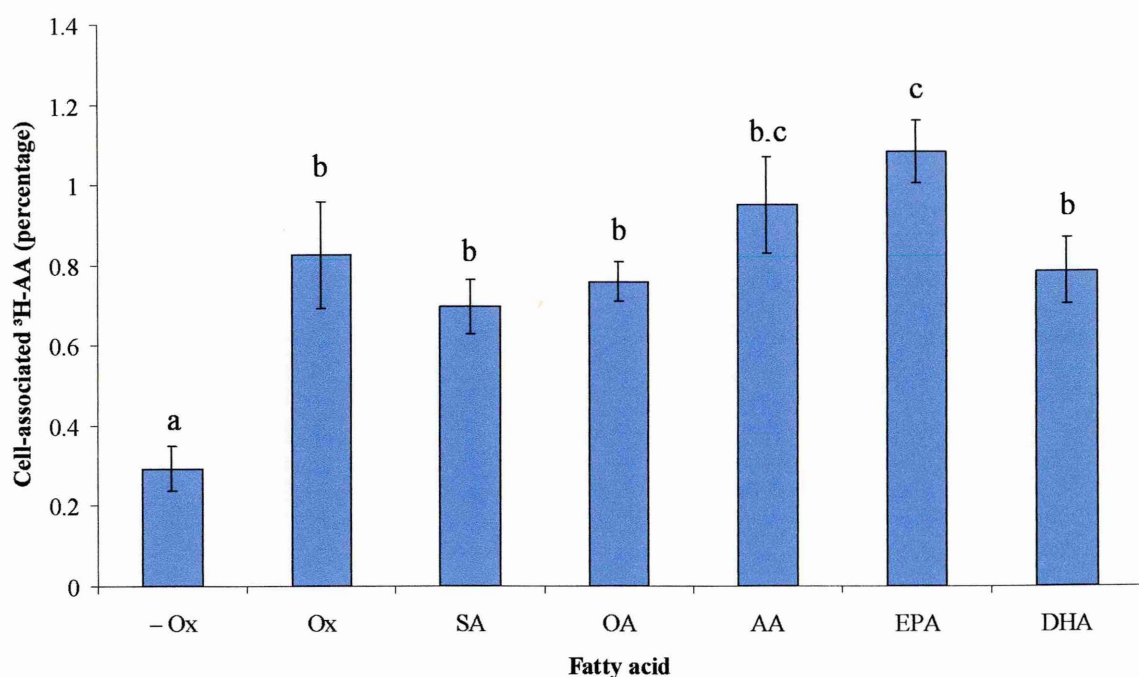
Unlike AA release from U937 cells treated with 10  $\mu$ M levels of the FAs for 24 h, the data shown in Fig. 6.5, demonstrate a markedly significant <sup>3</sup>H-AA release with EPA compared to all other treatments (One-way ANOVA  $F = 214.47$ ,  $P < 0.01$ ,  $DF = 6$ ). As expected, there was a significant difference between oxidant-untreated controls (– Ox) and treatments with the oxidant (750  $\mu$ M t-BHP/ $Fe^{2+}$ ). With continuous supplementation of U937 cells with the fatty acids for 18 days, one might expect to find a release not only of the radiolabelled fatty acid but also of the supplemented fatty acid independent of its degree of unsaturation, thus significantly evening out <sup>3</sup>H-AA release in all treatments. To address this possibility, cells were labelled with 0.1  $\mu$ Ci/ml <sup>3</sup>H-AA just 24 h prior to exposure to 750  $\mu$ M t-BHP/ $Fe^{2+}$ . However, oxidation induced no significant differences between oxidized controls (Ox) and SA or OA (Fig. 6.5). Again, there was a significant increase in <sup>3</sup>H-AA release with PUFA (AA, EPA or DHA) -

pretreatments compared to oxidant-treated controls (Ox). Surprisingly, the influence of prolonged EPA treatments of U937 cells produced a striking increase in AA release, significantly higher than that released from all other treatments including AA and even DHA. There was however no significant difference observed in  $^3\text{H}$ -AA released from incubations between cells pre-treated with AA or DHA for 18 days.

Since control experiments involving FA-pretreated cells alone for 18 days without oxidation were not included in this study, this experiment does not confirm the speculation that prolonged EPA treatment on its own stimulates AA release without oxidant-activation. It is important to conduct further studies to answer this question concerning the unexpected results (Fig. 6.5).

#### ***6.3.1.1 Cell-associated free fatty acids***

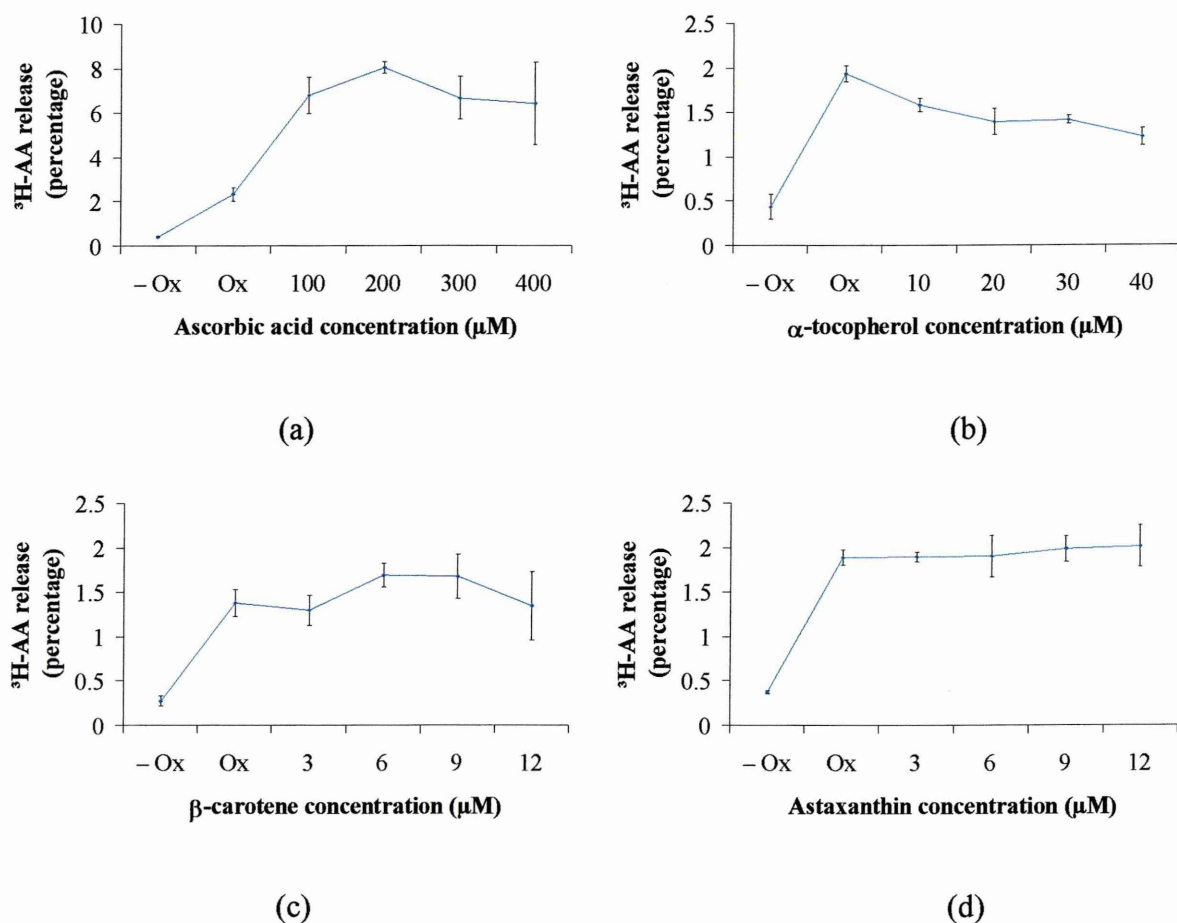
The amount of cell-associated free  $^3\text{H}$ -AA upon oxidative stimulation showed no significant differences between SA/OA/AA treatments and oxidized controls (Fig. 6.6). There was a significant difference observed between oxidized controls (Ox) and EPA treated cells only (One-way ANOVA  $F = 38.21$ ,  $P < 0.01$ ,  $DF = 6$ ). Neither was there any significant difference between AA and EPA nor AA and DHA. However, a significant difference between EPA and DHA treatments was found. The level of free  $^3\text{H}$ -AA associated with the cells (not incorporated into phospholipids) was in agreement with its release into the medium, particularly with EPA treatments compared to oxidant-treated controls (Fig. 6.6).



**Fig. 6.6** Cell-associated free fatty acids in FA-enriched U937 cells treated with 750  $\mu\text{M}$  of t-BHP/ $\text{Fe}^{2+}$  for 4 h. Means  $\pm$  95% CIs of two independent determinations ( $n = 4$ ). Letters: a, b and c denote significant differences between means ( $P < 0.01$ ).

### 6.3.2 With antioxidants

Given the high capacity of U937 cells to release  $^3\text{H}$ -AA into the extracellular fluid upon induced-oxidative stress, the question of whether pre-treatment with antioxidants inhibited this response was examined. For this purpose, U937 cells were treated initially with or without antioxidants and  $^3\text{H}$ -AA for 24 h prior to oxidant-induced  $^3\text{H}$ -AA release experiments. The presence of ascorbic acid at 100 – 400  $\mu\text{M}$  concentrations in U937 cells further increased  $^3\text{H}$ -AA release into the medium (Fig. 6.7a), suggesting that ascorbic acid enhanced pro-oxidative effects thereby stimulating AA mobilization rather than acting as an antioxidant, exerting suppressive effects on the oxidative process (One-way ANOVA  $F = 226.19$ ,  $P < 0.01$ ,  $DF = 5$ ).



**Fig. 6.7**  $^3\text{H-AA}$  release in U937 cells pre-treated for 24 h with varying antioxidants concentrations, before the exposure of cells to the oxidant mixture (a) With 0 – 400  $\mu\text{M}$  ascorbic acid; (b) with 0 – 40  $\mu\text{M}$   $\alpha$ -tocopherol; (c) with 0 – 12  $\mu\text{M}$   $\beta$ -carotene and (d) with 0 – 12  $\mu\text{M}$  astaxanthin. Data points represent the means  $\pm$  95% CIs ( $n = 4$ ).

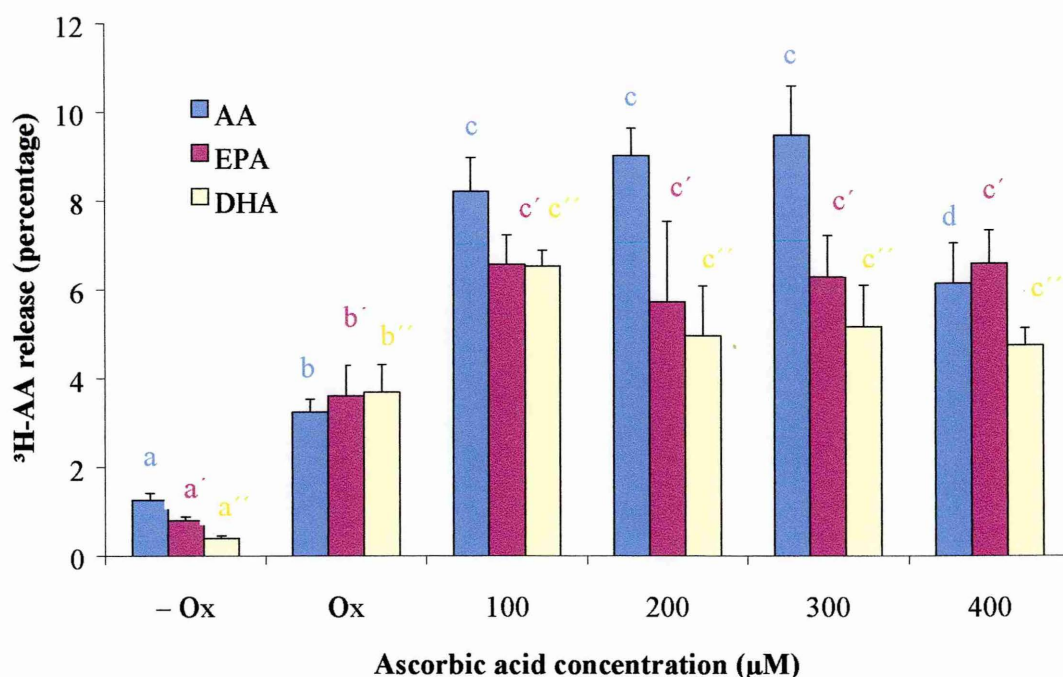
Ascorbic acid at concentrations  $\geq 100 \mu\text{M}$  in the cells generated a dramatic increase in the release of  $^3\text{H-AA}$  into the extracellular medium compared to oxidant-treated controls (Ox) after 4 h of incubation with  $750 \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ . (Fig. 6.7a). There was no significant difference between the effects of ascorbic acid at the concentration range used (100 – 400  $\mu\text{M}$ ). At 100  $\mu\text{M}$  ascorbic acid levels, there was 191% increase in AA release and 244% at 200  $\mu\text{M}$  compared to oxidized controls. The addition of 300  $\mu\text{M}$

yielded 185% increase in AA release and 175% at 400  $\mu\text{M}$ . Although, with increasing ascorbic acid levels (300–400  $\mu\text{M}$ ), there appears to be less deleterious oxidative effects, this decrease was not significantly different from other ascorbic acid concentrations.  $\alpha$ -tocopherol on the other hand, was a potent inhibitor of oxidant-induced AA release at a concentration  $\geq 10$   $\mu\text{M}$  to 40  $\mu\text{M}$  after 4 h of treating U937 cells with 750  $\mu\text{M}$  of the oxidant (One-way ANOVA  $F = 81.82$ ,  $P < 0.01$ ,  $DF = 5$ ) (Fig. 6.7b). At 10  $\mu\text{M}$   $\alpha$ -tocopherol levels an 18% reduction in AA release and 37% at 40  $\mu\text{M}$  compared to oxidized controls (Ox) were observed. Contrary to expectations, pre-treatment of cells with 0–12  $\mu\text{M}$  levels of the carotenoids,  $\beta$ -carotene (One-way ANOVA  $F = 40.67$ ,  $P < 0.01$ ,  $DF = 5$ ) (Fig. 6.7c) and astaxanthin (One-way ANOVA  $F = 142.13$ ,  $P < 0.01$ ,  $DF = 5$ ) (Fig. 6.7d), showed no inhibitory effects on AA release compared to oxidized controls. In all experiments (Fig. 6.7), oxidant-untreated controls (– Ox) generated significantly lower  $^3\text{H}$ -AA amounts than oxidant-treated controls (Ox).

Collectively, these results suggest that  $\alpha$ -tocopherol confers the highest antioxidant effects in U937 cells over prolonged oxidative stress (4 h) compared to ascorbic acid or even the carotenoids,  $\beta$ -carotene and astaxanthin, as measured by  $^3\text{H}$ -AA release into the extracellular fluid.

To further define the role of these antioxidants on  $^3\text{H}$ -AA release, the effects of adjunctive treatments with varying concentrations of the antioxidants (ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin), 10  $\mu\text{M}$  PUFAs (AA, EPA or DHA) and 0.1  $\mu\text{Ci/ml}$   $^3\text{H}$ -AA for 24 h pre-oxidation were examined in U937 cells. Fig 6.8 shows the effects of 0–400  $\mu\text{M}$  ascorbic acid on PUFA-enriched cells subjected to oxidation for 4 h under the condition stated earlier.





**Fig. 6.8** Induced-AA release in U937 cells treated with 10 μM PUFAs (AA, EPA or DHA) and various concentrations of ascorbic acid. Means ± 95% CIs (n = 4). Letters: a, b c and d represent significant differences between means at P < 0.01.

As shown in Fig. 6.8, ascorbic acid at 100 – 400 μM concentrations, promotes the pro-oxidative effects of t-BHP/Fe<sup>2+</sup> in PUFA-enriched U937 cells thus aggravating the effects already exerted by the oxidant system on its own. 100 – 400 μM ascorbic acid treatments further enhanced <sup>3</sup>H-AA release compared to oxidized controls (Ox). With 10 μM AA pre-treatment, there was a linear increase in AA release from 100 – 300 μM ascorbic acid concentrations after which it appears to reduce significantly at 400 μM level compared to other ascorbic acid concentrations (One-way ANOVA F = 112.68, P < 0.01, DF = 5). Results showed 153 – 193% increase in AA release with ascorbic acid concentrations ranging from 100 – 300 μM and 89% with 400 μM levels compared to oxidized controls (Ox). These results suggest a less toxic effect at 400 μM ascorbic acid levels in U937 cells co-treated with 10 μM AA. However, all ascorbic acid

treatments resulted in significantly higher AA release compared to both unoxidized (– Ox) and oxidized (Ox) controls.

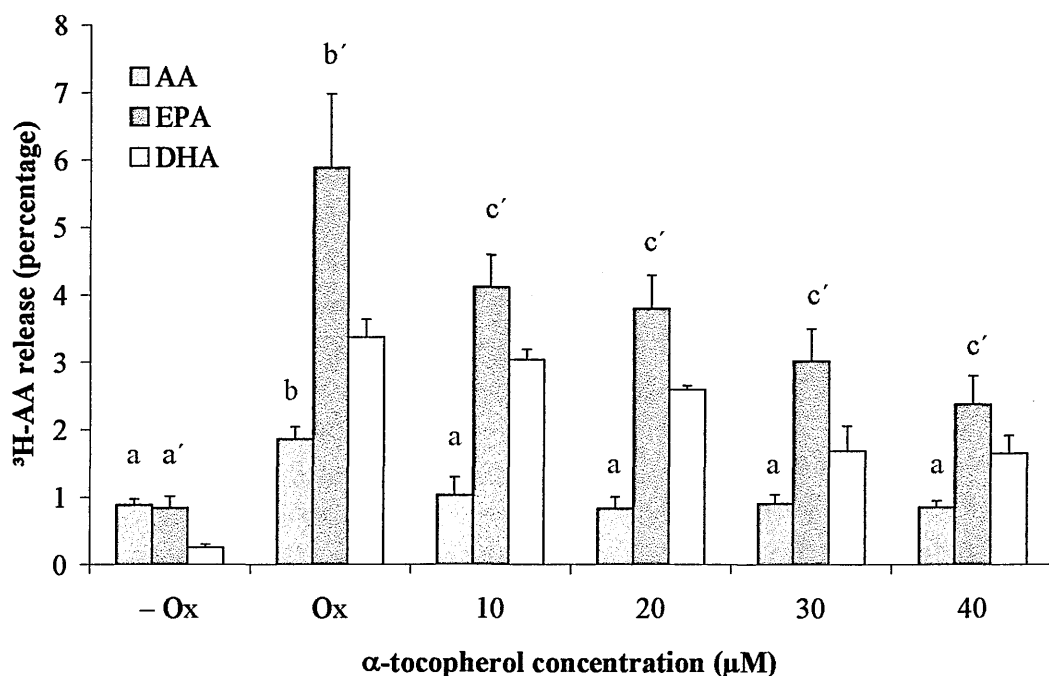
Neither the presence of EPA nor DHA in U937 cells influenced the damaging effects of ascorbic acid at 0 – 400  $\mu$ M levels. With both EPA and DHA treatments, there was a linear increase in AA release from unoxidized controls (– Ox) to 100  $\mu$ M ascorbic acid concentration, thereafter it began to plateau. With 10  $\mu$ M EPA treatments, there was an average increase of 74% in  $^3$ H-AA release with ascorbic acid concentrations ranging from 100 – 400  $\mu$ M compared to oxidant-treated controls (One-way ANOVA  $F = 41.17$ ,  $P < 0.01$ ,  $DF = 5$ ). With 10  $\mu$ M DHA, ascorbic acid levels at 100 – 400  $\mu$ M concentrations did not exert antioxidant properties in cells upon oxidation (One-way ANOVA  $F = 69.24$ ,  $P < 0.01$ ,  $DF = 5$ ). At 100  $\mu$ M ascorbic acid levels, there was 77% increase in AA release compared to oxidized controls, whereas there was a 29% increase with 400  $\mu$ M levels.

Taken together, it appears that the choice of PUFAs influence the activity of ascorbic acid. With DHA, ascorbic acid at 200 – 400  $\mu$ M exerted neither aggravating nor inhibitory properties on AA release observed in U937 cells. On the other hand, for EPA treatments, 100 – 400  $\mu$ M ascorbic acid treatments contributed to the pro-oxidative properties of the oxidant used hence generating  $^3$ H-AA release significantly greater than oxidized controls (Ox). There was a significant difference in  $^3$ H-AA release with AA pre-incubations co-treated with 100 – 400  $\mu$ M concentrations of ascorbic acid compared to controls. This results suggest that the presence of EPA and AA contribute more to the pro-oxidative properties of ascorbic acid than DHA in U937 cells, to varying extents, in the following order of magnitude AA > EPA > DHA. Even though these set of experiments were not replicated, it remains pertinent that ascorbic acid (100 – 400



$\mu\text{M}$ ) enhances  $^3\text{H}$ -AA release from U937 cells exposed to oxidative stress by 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ .

The effects of varying concentrations of the lipid-soluble antioxidant,  $\alpha$ -tocopherol, in U937 cells enriched with PUFAs were investigated (Fig. 6.9).



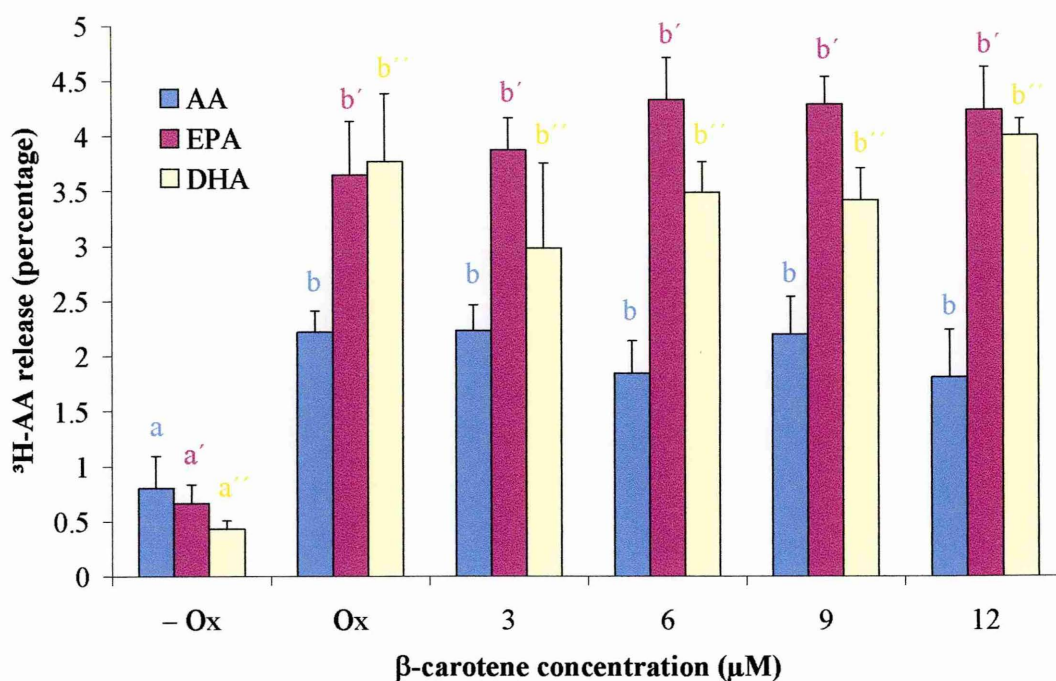
**Fig. 6.9** Effects of  $\alpha$ -tocopherol concentrations ranging from 0 – 40  $\mu\text{M}$  levels and PUFA enrichment on  $^3\text{H}$ -AA release in U937 cells. Means  $\pm$  95% CIs ( $n = 4$ ). Letters: a, b, c and d indicate significant differences between means at  $P < 0.01$ .

To determine the role of  $\alpha$ -tocopherol on *in vitro* oxidant-induced  $^3\text{H}$ -AA release by t-BHP/ $\text{Fe}^{2+}$  post-PUFA enrichment, U937 cells were pre-treated with 0 – 40  $\mu\text{M}$   $\alpha$ -tocopherol levels and 10  $\mu\text{M}$  AA, EPA or DHA for 24 h prior to the induction of oxidative stress (Fig. 6.9). With AA treatment, there was a significantly lower  $^3\text{H}$ -AA release observed at 10  $\mu\text{M}$   $\alpha$ -tocopherol levels compared to oxidized controls (One-way ANOVA  $F = 17.83$ ,  $P < 0.01$ ,  $DF = 5$ ). In fact, at  $\alpha$ -tocopherol concentrations  $\geq 10$   $\mu\text{M}$ , there was no significant difference between oxidant-untreated controls (– Ox),

suggesting a potent inhibitory role of  $\alpha$ -tocopherol on AA-pre-treated U937 cells. EPA and DHA treatments generated similar results with  $^3\text{H}$ -AA release significantly inhibited at  $\alpha$ -tocopherol levels  $\geq 10 \mu\text{M}$ , compared to oxidant-treated controls (Ox): EPA (One-way ANOVA  $F = 47.01$ ,  $P < 0.01$ ,  $DF = 5$ ); DHA (One-way ANOVA  $F = 128.22$ ,  $P < 0.01$ ,  $DF = 5$ ). With  $10 \mu\text{M}$  EPA treatments, there was 30% decrement in  $^3\text{H}$ -AA release at  $10 \mu\text{M}$   $\alpha$ -tocopherol concentration and 60% at  $40 \mu\text{M}$  levels when compared to oxidant-treated controls (One-way ANOVA  $F = 47.01$ ,  $P < 0.01$ ,  $DF = 5$ ). In DHA pre-treated cells, results demonstrated 10% inhibitory effects of  $\alpha$ -tocopherol at  $10 \mu\text{M}$  concentrations and 51% at  $40 \mu\text{M}$  compared to oxidized controls.

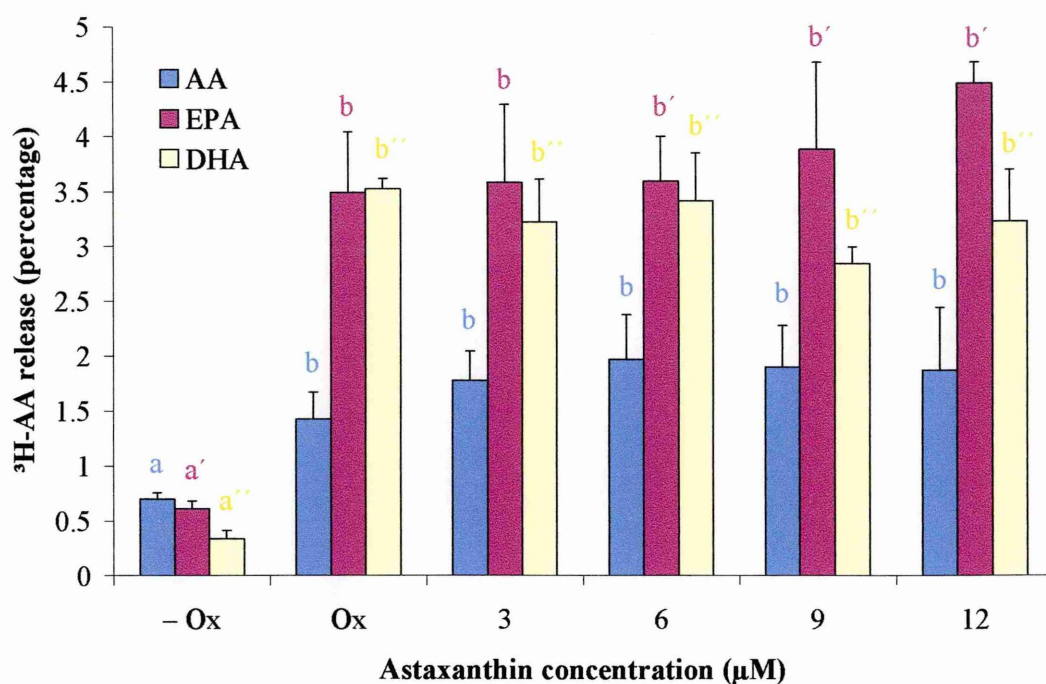
Overall,  $\alpha$ -tocopherol conferred inhibitory effects on PUFA-treated U937 cells at concentrations  $\geq 10 \mu\text{M}$  levels as measured by extracellular  $^3\text{H}$ -AA release. Its potency was greater in the presence of PUFA in the order: AA > EPA > DHA when compared to their respective oxidant-treated controls (Ox).

To attempt to inhibit  $^3\text{H}$ -AA release induced by t-BHP/ $\text{Fe}^{2+}$ , U937 cells co-treated with carotenoids,  $\beta$ -carotene (Fig. 6.10) or astaxanthin (Fig. 6.11), and  $10 \mu\text{M}$  PUFA for 24 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in a humid environment were examined. Fig. 6.10 shows the effect of 0 –  $12 \mu\text{M}$   $\beta$ -carotene concentrations on  $^3\text{H}$ -AA release. With AA (One-way ANOVA  $F = 13.75$ ,  $P < 0.01$ ,  $DF = 5$ ), EPA (One-way ANOVA  $F = 105.64$ ,  $P < 0.01$ ,  $DF = 5$ ) and DHA (One-way ANOVA  $F = 65.12$ ,  $P < 0.01$ ,  $DF = 5$ ) treatments,  $\beta$ -carotene at 3 –  $12 \mu\text{M}$  levels manifested no protective antioxidant role in U937 cells. With all three PUFA treatments, there was no significant difference between oxidant-treated controls (Ox) and 3 –  $12 \mu\text{M}$   $\beta$ -carotene levels but as expected there was a significantly lower AA release from unoxidized controls (– Ox) when compared to all treatments involving the oxidant (Fig. 6.10).



**Fig. 6.10** Oxidant-induced  $^3\text{H}$ -AA release into the extracellular medium of U937 cells treated with 10  $\mu\text{M}$  PUFA (AA, EPA or DHA) and  $\beta$ -carotene concentrations ranging from 0 – 12  $\mu\text{M}$ . Means  $\pm$  95% CIs ( $n = 4$ ). Letters: a and b represent significant differences between means at  $P < 0.01$ .

In a like manner, as shown in Fig. 6.11, treatments of PUFA-enriched U937 cells with 0-12  $\mu\text{M}$  astaxanthin concentrations did not result in the inhibition of  $^3\text{H}$ -AA release into the extracellular fluid after 4 h of induced oxidative stress at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in a humid environment: AA treatment (One-way ANOVA  $F = 9.65$ ,  $P < 0.01$ ,  $\text{DF} = 5$ ); EPA (One-way ANOVA  $F = 46.84$ ,  $P < 0.01$ ,  $\text{DF} = 5$ ) and DHA (One-way ANOVA  $F = 116.78$ ,  $P < 0.01$ ,  $\text{DF} = 5$ ). With 10  $\mu\text{M}$  AA, EPA or DHA, there was a significant increase in oxidant-treated controls (Ox) compared to unoxidized controls (- Ox). There was no significant difference observed between oxidized controls and 3 – 12  $\mu\text{M}$  astaxanthin treatments (Fig. 6.11).



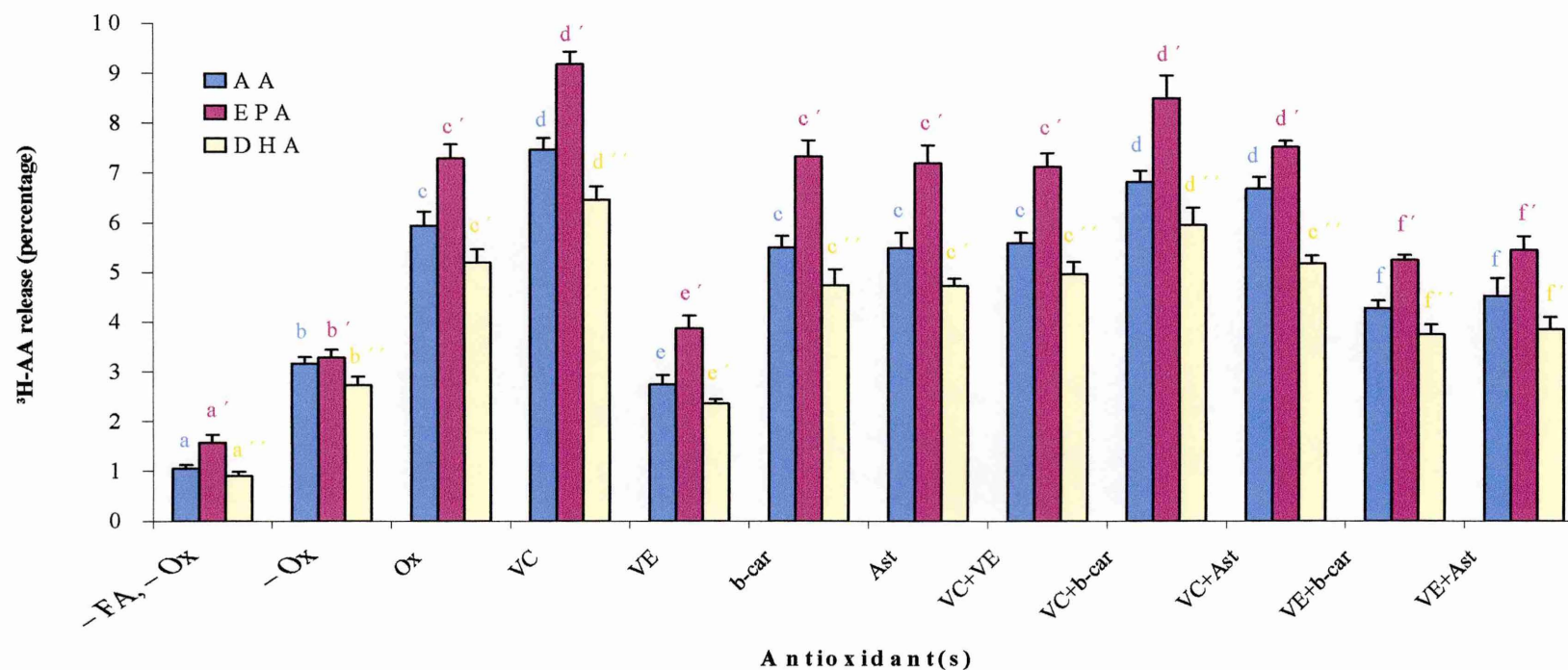
**Fig. 6.11** Oxidant-induced  $^3\text{H}$ -AA release from U937 cells treated with PUFAs (AA, EPA or DHA) and astaxanthin concentrations ranging from 0 – 12  $\mu\text{M}$ . Means  $\pm$  95% CIs ( $n = 4$ ). Letters: a and b denote significant differences between means at  $P < 0.01$ .

The next series of experiments sought to determine whether it was possible to further suppress the release of  $^3\text{H}$ -AA into the extracellular fluid under the conditions stated earlier. To this end, the effects of several antioxidant combinations in PUFA-rich U937 cells stimulated by the oxidant mixture, were examined. U937 cells were pre-treated with 10  $\mu\text{M}$  AA, EPA or DHA coupled with antioxidants (individuals and mixtures) for 24 h after which the cells were challenged with 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for 4 h. The final antioxidant concentrations used in CM were 400  $\mu\text{M}$  ascorbic acid, 40  $\mu\text{M}$   $\alpha$ -tocopherol, 12  $\mu\text{M}$   $\beta$ -carotene or astaxanthin on all occasions when used either individually or as combinations.

In order to determine whether the activation of  $^3\text{H}$ -AA release in U937 cells by oxidative stress stimuli is suppressed by pre-treatment of cells with a combination of antioxidants, the possible effects of individual and mixtures of antioxidants on PUFA-enriched cells were also examined (Fig. 6.12). The data in Fig. 6.12 clearly demonstrates that  $\alpha$ -tocopherol on its own was the most effective antioxidant against the induced release of  $^3\text{H}$ -AA significantly inhibiting the amount of  $^3\text{H}$ -AA released into the medium compared with oxidant-treated controls (Ox): AA (One-way ANOVA  $F = 451.77$ ,  $P < 0.001$ ,  $DF = 11$ ); EPA (One-way ANOVA  $F = 340.34$ ,  $P < 0.001$ ,  $DF = 11$ ) and DHA (One-way ANOVA  $F = 267.68$ ,  $P < 0.001$ ,  $DF = 11$ ). Vitamin C (ascorbic acid) significantly increased AA release compared to oxidized controls. When ascorbic acid was combined with one other antioxidant, the presence of  $40\ \mu\text{M}$   $\alpha$ -tocopherol or  $12\ \mu\text{M}$  astaxanthin but not  $12\ \mu\text{M}$   $\beta$ -carotene reduced the contributory pro-oxidative effects observed with ascorbic acid alone, though not significantly different from that observed in oxidant-treated controls (Ox).

Studies also investigated whether the combination of antioxidants generated additive or synergistic inhibition in the release of  $^3\text{H}$ -AA from U937 cells particularly combinations of  $\alpha$ -tocopherol with astaxanthin or  $\beta$ -carotene. Contrary to expectations, although there was a significant reduction in  $^3\text{H}$ -AA release with cells pre-treated with  $\alpha$ -tocopherol and either  $\beta$ -carotene or astaxanthin, the reduction was not as significant as that observed with  $\alpha$ -tocopherol on its own when compared to oxidant-treated controls (Fig. 6.12). These data therefore suggest that the presence of astaxanthin or  $\beta$ -carotene somehow interferes with the activities of  $\alpha$ -tocopherol, thus reducing the sole inhibitory effects of  $\alpha$ -tocopherol conferred on U937 cells. It also demonstrates that either of these two carotenoids can act also as pro-oxidants, especially under prolonged oxidative stress such as that used in this study (t-BHP/ $\text{Fe}^{2+}$  for 4 h).

As previously shown, there was no significant difference between oxidant-treated controls (Ox) and treatments with 12  $\mu$ M  $\beta$ -carotene or 12  $\mu$ M astaxanthin. With adjunctive treatments of cells with both ascorbic acid and  $\alpha$ -tocopherol, although there was no conferred pro-oxidative influence contributing to the release of AA by ascorbic acid, neither was there observed any inhibitory effect of  $\alpha$ -tocopherol compared to oxidized controls, demonstrating that the latter exerted its antioxidant properties by inhibiting further peroxidative damage by ascorbic acid. As such AA release in cells pre-treated with  $\alpha$ -tocopherol and ascorbic acid was not significantly different from oxidant-treated controls (Fig. 6.12).  $^3\text{H}$ -AA release from oxidant-untreated control cells (– Ox) was markedly different at  $P < 0.01$  from all other treatments studied.



**Fig. 6.12** Effects of individual and mixtures of antioxidants on oxidant-induced release of  $^3\text{H-AA}$  in U937 cells pre-treated with  $10\ \mu\text{M}$  AA, EPA or DHA. Means  $\pm$  95% CIs ( $n = 4$ ). Where: (- FA, - Ox) represents blank experiments without PUFA and oxidant; - Ox: controls with PUFA but without oxidant, Ox: with both PUFA and oxidant, VC: ascorbic acid, VE:  $\alpha$ -tocopherol, b-car:  $\beta$ -carotene and Ast: astaxanthin. All experiments were pre-incubated with  $^3\text{H-AA}$  and those involving antioxidants were all exposed to the oxidants. Letters: a, b, c, d, e and f indicate significant differences between treatments at  $P < 0.001$ .



## 6.4 DISCUSSION AND CONCLUSION

The objective of this study was to investigate whether a relationship exists between oxidative stress and AA release into the extracellular fluid using the human lymphoma cell line U937 as a model. Following the establishment of coupling between these two processes, the effects of cellular pre-treatment with the PUFAs (AA, EPA or DHA) as well as varying concentrations of the dietary antioxidants (ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin) both individually and as mixtures were evaluated.

Monocytic cells are naturally capable of producing highly reactive oxygen species such as superoxide anion and hydrogen peroxide in response to a variety of stimulants (Lum and Roebuck, 2001). Although these metabolites play critical roles *in vivo* or *in vitro* in inflammatory processes such as host defense, chemotaxis and exocytosis of lysosomal contents (Snyderman and Pike, 1984; Hugli, 1984; Fisher et al., 1995), their production in uncontrolled amounts may lead to serious cellular dysfunctions. Oxidative injury (Mahadik et al., 2001), elevated phospholipid breakdown (Ross et al., 1997) and increased PLA<sub>2</sub> activity (Gattaz et al., 1995) are often implicated in schizophrenia. Also, arachidonic acid release has been associated with oxidative damage in several cell types such as the vascular smooth muscles, stromal cells, striatal neurons, platelets and phagocytes (Chakraborti and Chakraborti, 1995; Rao et al., 1995; Boyer et al., 1995; Samanta et al., 1998; Buschbeck et al., 1999; Birbes et al., 2000).

In this study, a mixture of tert-butyl hydroperoxide and iron sulfate (t-BHP/Fe<sup>2+</sup>) was used to investigate the release of arachidonic acid in U937 cells subjected to oxidative stress, and the data suggest that AA mobilization from cellular phospholipids is both dose – and time – dependent on the oxidant. This study shows that oxidant-induced AA release increases with increasing concentration of the PUFAs (AA, EPA or DHA) but



not SA or OA as previously reported by Robinson et al. (1998). Examination of the time course release of  $^3\text{H}$ -AA in response to BHP/ $\text{Fe}^{2+}$  revealed that after an initial time lag of 3 h, the response proceeded linearly with time, showing signs of saturation after exposure of cells to the oxidant mixture for 5 h.

The role of trace redox active transition metals such as iron is essential in many biological reactions such as the synthesis of DNA, RNA and proteins, and also as co-factors of numerous enzymes, particularly those involved in respiration. The lack of these metals may therefore lead to dysfunctions in the CNS and other organ functions but their presence in excessive amounts can be cytotoxic, due to oxidative stress and increased free radical production as shown in this study. Macrophage-like cells in the CNS (microglia) are the major sites of ferritin bound iron and are thought to be partly responsible for oxidative damage in Parkinson's disease (Sayre et al., 1999).

Saturated and mono-unsaturated fatty acids can be synthesized by the human body *de novo*. However, since mammals cannot introduce double bonds beyond the delta-9 position in the fatty acid chain, linoleic and linolenic acid, the n-6 and n-3 series precursors respectively, must be supplied by the diet. Based on this, the effects of oxidation on AA released into the medium after the enrichment of U937 cells with various concentrations of different fatty acids (SA, OA, AA, EPA or DHA) ranging from 10 – 40  $\mu\text{M}$ , were determined. Pre-incubation with these concentrations of SA or OA generated no significant differences in AA released into the extracellular fluid compared to oxidant-treated controls. Increased oxidant-induced AA release however appeared to be a general feature with increasing concentrations of the PUFAs (AA, EPA and DHA).

In order to investigate the possible involvement of cPLA<sub>2</sub> in the release of incorporated <sup>3</sup>H-AA, U937 cells, cells were cultured with equal amounts of the fatty acids SA, OA, AA, EPA or DHA in one single experiment (Fig. 6.4), before the induction of oxidative stress with t-BHP/Fe<sup>2+</sup>. Results demonstrated that neither SA nor OA, nor AA released <sup>3</sup>H-AA in an amount significantly different from controls whereas, EPA and DHA did. However, post-oxidation (Fig. 6.3) data showed whilst there was no significant difference between SA or OA in comparison to oxidized controls, there were significant differences with PUFA-treated cells following 24 h of cellular pre-treatments in the following order: AA < DHA < EPA. When U937 cells were consecutively treated with these fatty acids over 18 days, a striking difference in AA release was observed between the PUFAs with the greatest increase observed with EPA. A possible explanation for this marked increase in AA release with EPA – and not DHA – pre-treatment after continuous PUFA supplementation is that upon oxidative stress, the presence of EPA in U937 cells, results in a rapid accumulation of cell-associated free <sup>3</sup>H-AA preceding its mobilization into the incubation medium over time, as suggested from Fig. 6.6, by a mechanism yet to be investigated. Whether certain gene expressions were altered by EPA but not DHA over the 18 days of continuous supplementation, thereby stimulating such markedly higher AA release upon oxidation, is unknown at present. Collectively, these results suggest that oxidative stress activates a PLA<sub>2</sub> enzyme in PUFA-enriched U937 cells, which in turn triggers the release of AA from cell membrane phospholipids.

AA is almost exclusively located at the sn-2 position of phospholipids, and some of the several pathways postulated for its release include: the direct hydrolysis of a phospholipase A<sub>2</sub> (Bills et al., 1976; Billah and Lapetina 1982); hydrolysis by a phospholipase A<sub>1</sub> followed by the action of a lysophospholipase (Van den Bosch, 1980; Irvine, 1982; Emilsson and Sundler, 1984); lipolysis by a PI-specific PLC followed by

the activity of a DAG lipase (Mauco et al., 1978; Bell et al., 1979; Rittenhouse-Simmons, 1979) or the sequential action of PLC, DAG kinase, and a phosphatidic acid specific PLA<sub>2</sub> (Lapetina et al., 1981; Billah et al., 1981). These proposed mechanisms are not inhibitory of one another and may occur simultaneously.

Among the different known types of phospholipase A<sub>2</sub> enzymes, cPLA<sub>2</sub>, apart from occurring in high quantities (Rzizgalinski et al., 1996), is considered as the key intracellular enzyme responsible for the release of AA in stimulated U937 cells (Kramer, 1994). Furthermore, Hsu et al. (2000) reported that changes in membrane lipid composition promote cPLA<sub>2</sub> activation in differentiated U937 cells in a manner requiring neither the phosphorylation of the enzyme nor alterations in its level. In contrast to these observations, Balboa and Balsinde (2002), recently implicated iPLA<sub>2</sub> in hydrogen peroxide-induced AA release from U937 cells because treatment of the cells with bromoenol lactone, a compound with marked selectivity for inhibiting iPLA<sub>2</sub>, resulted in a significant reduction in H<sub>2</sub>O<sub>2</sub>-activated AA release.

Since all three forms of PLA<sub>2</sub>s (Ca<sup>2+</sup>-dependent/ independent cPLA<sub>2</sub> and iPLA<sub>2</sub>) occur in U937 cells and it is well known that cPLA<sub>2</sub> and not iPLA<sub>2</sub> has a preference for AA at its sn-2 position, this conclusion argues against the possibility that iPLA<sub>2</sub> is solely responsible for the mobilization of AA from H<sub>2</sub>O<sub>2</sub>-treated U937 cells. Perhaps the treatment of U937 cells with H<sub>2</sub>O<sub>2</sub> sends a different signal to these cells thus activating AA release via a mechanism involving iPLA<sub>2</sub> and not cPLA<sub>2</sub> activation since these cells naturally produce H<sub>2</sub>O<sub>2</sub> in response to a variety of agonists (Lum and Roebuck, 2001). Since AA is preferably located esterified at the sn-2 position of cellular phospholipids, a PLA<sub>2</sub> is the key enzyme responsible for regulating the levels of free fatty acids (Dillon et al., 1997). However free AA levels available for eicosanoid synthesis represent a

balance between the amount liberated by the activated PLA<sub>2</sub> minus what is re-incorporated back into phospholipids by the acyltransferase enzymes (Chilton et al., 1996). Therefore, the levels of available free fatty acids are efficiently controlled by the reacylation pathway as well (Chilton et al., 1996). Taking all these findings together, more work is required to investigate oxidant-stimulated AA release in U937 cells.

The differences observed in the release of <sup>3</sup>H-AA from U937 cells pre-treated with the polyunsaturated fatty acids: arachidonic, eicosapentaenoic and docosahexaenoic acids, will be discussed in more detail at the end of this study (Chapter nine).

#### **6.4.1 With antioxidants**

Despite the fact that vitamin E ( $\alpha$ -tocopherol) is a powerful hydrogen atom donor towards free radicals, and particularly peroxyl radicals, it does not function as a generalized biological reducing agent, although its phenolate ion may reduce some transition metals in certain situations. In contrast, vitamin C (ascorbic acid) is an essential co-factor and an important biological reducing agent for iron, for instance. Vitamin C acts as a general reducing agent toward a variety of oxidants, including supplying reducing equivalents in enzymatic reactions. Although it is truly one of the important water-soluble antioxidants, it is not unique in this role. Other hydrophilic antioxidants include uric acid (Peden et al., 1990), and glutathione (Halliwell and Gutteridge, 1999). Therefore vitamin C, in contrast to vitamin E, has many other well-established functions in addition to being an antioxidant, which is not its unique role. Vitamin E is the most important lipid-soluble peroxyl radical scavenger in human cells and acting as an antioxidant clearly is one of its principal roles and quite possibly the most important (Pryor, 2000). Both vitamins E and C have an aromatic hydroxyl group

capable of acting as an electron donor whereas carotenoids such as  $\beta$ -carotene is a hydrocarbon, and therefore a poor structural choice as a general reducing agent.

In order to determine whether CM-supplementation with the dietary antioxidants (ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin) inhibited oxidant-induced AA release in U937 cells, this study first examined the possible inhibition of incorporated  $^3\text{H}$ -AA release by pre-treating the cells with the antioxidants alone for 24 h before oxidation (Fig. 6.7). This study demonstrates that ascorbic acid, an agent known to scavenge free radicals, also has the capacity to promote oxidative stress in U937 cells (Frei and Carr, 1999). Vitamin C interacts with redox active transition metal ions such as iron and copper. The reduction of these transition metals by ascorbate ion can have deleterious effects in biological systems by the production of alkoxyl and hydroxyl radicals promoting peroxidative damage (Halliwell, 1996; Buettner and Jurkiewicz, 1996). The relevance of this Fenton reaction has been a controversial issue *in vivo* because of the availability of the metal ions especially iron (Halliwell and Gutteridge, 1986). Although free metal ions are thought to exist in low concentrations *in vivo* due to their presence in sequestered forms in various binding proteins such as ferritin and transferrin (Halliwell and Gutteridge, 1986), these ions may be released during tissue injury subsequently reacting with vitamin C (Halliwell, 1996) thereby escalating peroxidative processes resulting in damaging consequences to the cells involved (Fig. 6.7a).

$\alpha$ -tocopherol – treatment in animal models reduced the formation of thromboxanes in stimulated platelets (Hamelin and Chan, 1983) and prostaglandin  $\text{E}_2$  in activated macrophages (Sakamoto et al., 1991). Since AA release by  $\text{PLA}_2$  is the rate-limiting step in the biosynthesis of the eicosanoid hormones, the inhibition of  $\text{PLA}_2$  activity

(Douglas et al., 1986; Cao et al., 1987; Sakamoto et al., 1993) is generally referred to as the mechanism for the attenuation of the production of eicosanoids. In the present study, vitamin E inhibits the release of free AA reducing its availability for eicosanoid synthesis in oxidant-stimulated U937 cells, possibly by acting as a potent antioxidant. In contrast, vitamin E enrichment has also been shown to stimulate eicosanoid synthesis in some other cell types by causing an increase in AA release in cultured endothelial cells (Tran and Chan, 1988); rat heart myoblastic cells (H9c2) (Tran et al., 1996) and prostaglandin I<sub>2</sub> synthesis (Tran and Chan, 1990). The apparent difference in the activity of  $\alpha$ -tocopherol between mammalian cell types illustrates the fragmented nature of the understanding of the contributions of vitamin E in the regulation of arachidonic acid release with the knowledge of its specific mode of actions still remaining an elusive concept.

Considerable data suggest that excessive oxidative injury may be involved in the development of neurodegenerative diseases such as schizophrenia (Mahadik et al., 2001), Alzheimer's disease (Markesbury, 1997) and Parkinson's disease (Ebadi et al., 1996). Vitamin E intake has been reported to show protective roles against the occurrence of schizophrenia (Dorfman-Etrog et al., 1999), Alzheimer's disease (Sano et al., 1997) and Parkinson's disease (de Rijk et al., 1997). Vitamin E has also been shown to protect neurons from the effects of certain neurotoxic agents such as those from estradiol-induced neurotoxicity (Desjardins et al., 1992). Just like  $\alpha$ -tocopherol, the carotenoids are lipid-soluble substances and potent scavengers of free radicals. However, neither  $\beta$ -carotene nor astaxanthin exhibited any protective antioxidant effects against AA released after 4 h of induced-oxidative stress in U937 cells. Whilst the enrichment of U937 cells with  $\alpha$ -tocopherol led to a marked inhibition of AA release into the incubation medium, ascorbic acid enhanced its release whereas the carotenoids

showed no significant effects. This study supports the suggestion that  $\alpha$ -tocopherol may be the major chain breaking antioxidant during cell-mediated oxidation (Reaven et al., 1993; Diaz et al., 1997).

It is possible that the antioxidant effectiveness of  $\beta$ -carotene or astaxanthin was not observed because the concentration used in this study, pertaining to the oxidation time (4 h), was too small to elicit an effect. This assumption is based on the antioxidant effects observed with  $\beta$ -carotene and astaxanthin in chapter two after 30 minutes of induced-oxidation. Or perhaps the nature of *in vitro* enrichment of U937 cells as micelles (in chloroform) may have resulted in the incorporation of the carotenoids in a fashion dissimilar to that normally obtained *in vivo* (Borel et al., 1996), thus masking the true antioxidant effects of these carotenoids. Furthermore, the carotenoid radical intermediate formed in the process of breaking lipid peroxidative chain reactions, may have contributed to the propagation of oxidation (Dugas et al., 1998) rather than breaking the chain just like  $\alpha$ -tocopherol under certain conditions (Stocker et al., 1991; Bowry et al., 1992). It was demonstrated that  $\alpha$ -tocopherol affects the structure and dynamic properties of membranes (Srivastava et al., 1983; Suzuki et al., 1993). Several studies have suggested that interactions between vitamin E and membranes play a critical role in its mechanisms of antioxidant action and stabilization of membranes (Gomez-Fernandez et al., 1989). At high concentrations, vitamin E can have destructive effects on membranes under the “electric” (tensed) environment found in living cell membranes, probably the reason for its low concentrations in biological membranes (Koronkiewicz et al., 2001).

#### 6.4.2 Interaction between antioxidants

In an attempt to characterize this observation further, this study investigated whether the inhibitory effects of the dietary antioxidants (ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin) would be more effective in combinations in PUFA-treated cells (Fig. 6.12). Of the four different dietary antioxidants used individually, only  $\alpha$ -tocopherol conferred protective inhibitory effects on U937 cells by suppressing AA release. Nonetheless, because  $^3\text{H}$ -AA release is inhibited by the presence of  $\alpha$ -tocopherol in U937 cells, the effects of single and mixtures of antioxidants on its release, were compared. Oxidant-mediated  $^3\text{H}$ -AA release observed in U937 cells enriched with the dietary antioxidants (both individual and mixtures) is quite striking for two reasons: vitamin E single-handedly showed the greatest potency against the oxidant whereas when in combination with  $\beta$ -carotene or even astaxanthin, these appeared to interfere with its actions making it less effective.

Ascorbic acid treatment further contributed to the peroxidative damage observed by enhancing AA release from U937 cells. It is known that antioxidants influence one another by biological interactions. For example, a combination of vitamin C with vitamin E was reported to exert synergistic effects in schizophrenia (Kanofsky and Sandyk 1992). Also, Kanofsky and Sandyk (1992) previously reported a synergistic effect between vitamins C and E in schizophrenia ameliorating the chronic symptoms observed in patients. In contrast, the present study demonstrates that vitamin C promotes AA release in t-BHP/ $\text{Fe}^{2+}$  - treated U937 cells but when used in combination with vitamin E, there is a reduction in the damage that would have been done by vitamin C. The effects of combining antioxidants as a measure of suppressing lipid peroxidation has previously been investigated in order to determine whether interactions exist between them. Palozza and Krinsky suggested some cooperation between



tocopherols and the carotenoids (Paloza and Krinsky, 1991; 1992). They showed that synergy occurs between  $\beta$ -carotene and  $\alpha$ -tocopherol with the latter having a protective role over the former. On the other hand, a protective or reparative role of the carotenoids over  $\alpha$ -tocopherol in response to lipid peroxidation was also suggested in chicks (Mayne and Parker, 1989) and low-density lipoproteins (Packer, 1993). Ascorbic acid was found to protect  $\alpha$ -tocopherol and not  $\beta$ -carotene in a fatty acid methyl ester, producing additive antioxidant effects against oxidation (Vile and Winterborne, 1988; Niki et al., 1995a).

#### 6.4.3 Conclusion

In conclusion this chapter demonstrates that induced oxidative stress with t-BHP/ $\text{Fe}^{2+}$  stimulates arachidonic acid release in a time- and dose-dependent manner from U937 cells. It also shows that some PUFAs especially EPA, stimulate AA release with or without oxidation. Oxidant-mediated AA release can be significantly suppressed by pre-treating these cells with  $\alpha$ -tocopherol. Depending on the conditions under which a system is monitored, all antioxidants can also exert pro-oxidative effects.

It is however important to extend these studies to *in vivo* enrichments of carotenoids, ascorbic acid and  $\alpha$ -tocopherol individually and as combinations, in human subjects (however difficult this may be), to confirm the results presented herein. Overall the role of carotenoids as antioxidants in living systems requires much further understanding especially in terms of the underlying chemical and biochemical mechanisms of actions. Taken together, these results support a role for  $\text{PLA}_2$  – mediated AA release with oxidative stress. However, the particular  $\text{PLA}_2$  enzyme responsible for this activation requires further investigation.

## **CHAPTER 7: OXIDANT-INDUCED ARACHIDONIC ACID UPTAKE**

### **7.1 INTRODUCTION**

Membrane phospholipids are involved in apoptosis, regulation of enzyme activities, and generation of second messengers as well as serving as membrane anchors responsible for membrane fusion and antioxidants (Farooqui et al., 2000) such as plasmalogens, which protect PUFAs from iron-induced lipid peroxidative damage (Sindelar et al., 1999). Phospholipids are integral components of neuronal membranes and they exist in a dynamic flux. They are maintained by continuous synthetic and degradative processes. Apart from being a reservoir for second messengers, they may be involved in many important processes such as apoptosis in brain tissues (Fadok et al., 1992). Apoptosis is a form of programmed cell death characterized by nuclear condensation, cell shrinkage, and bleb formation. During this process, the cell plasma membrane rapidly translocates PS to the outer leaflet where it functions as a signal on the dying cell for recognition and removal by phagocytosis by an unknown specific mechanism (Fadok et al., 1992).

Saturated fatty acids particularly, palmitic and stearic, are usually located at the sn-1 position of the phospholipid molecule whereas PUFAs such as AA, EPA and DHA are esterified at the sn-2 position. Whilst saturated fatty acids appear to have a role in neuroplasticity (Tone et al., 1987; Wakabayashi et al., 1994), PUFAs contribute to membrane fluidity, playing crucial roles in the maintenance of the orientation of internal and external membrane regions for interactions amongst intracellular organelles, at the same time providing an appropriate environment for maximal functioning of membrane associated proteins such as receptors, membrane-bound enzymes and ion-channels (Farooqui et al., 2000). Comparative studies of fatty acid compositions of the different phospholipid classes in different cell types from the adult rat cerebral cortex indicated that FA acid compositions in phospholipids varies from cell to cell. Differences also

occur among the different phospholipid classes (Porcellati, 1983). Therefore, to determine membrane compositions *in vivo* under physiological conditions, it is necessary to investigate the phospholipid composition of a particular suborganelle of a specific tissue and not of membrane fractions that may have been contaminated by other organelles. Furthermore, PUFA release by the catalytic activity of cPLA<sub>2</sub> in response to agonists such as A23187 in U937 cells is specific for AA and EPA but not DHA (Rosenthal et al., 1995). Collectively, these observations indicate that AA, EPA and DHA, each influence different effects in biological systems.

PUFAs largely occur bound to serum proteins in biological systems. Protein binding is accompanied by rapid dissociation and exchange between the blood-brain barrier of unbound and unincorporated fatty acids (Banks et al., 1997; Fenton et al., 2000). Neural membranes are rich in PUFAs and so vulnerable to oxidative damage. Furthermore, the brain is rich in iron and surrounded by the cerebrospinal fluid, which contains little or no iron-chelating capacity. Since free radicals are generated during normal cellular processes (Halliwell, 1994), the fatty acid located at the sn-2 position is more susceptible to oxidative attack at the  $\alpha$ -methylene carbon atom adjacent to the carbon-carbon double bond (Farooqui et al., 2000).

Lipid hydroperoxides produced from such reactions are not completely stable *in vivo* and in the presence of iron can decompose further, propagating peroxidative chain reactions initiated in the first place by the free radical attack. They metabolise aldehydes, which can in turn cross-link enzymes and proteins making them inactive. Lipid peroxidative damage to neuronal membranes may lead to changes in physicochemical properties thus affecting the fluidity of these membranes and altering the orientation of optimal domains for the interaction of functional membrane proteins

(Farooqui et al., 2000). In addition, it may cause changes in the number of receptors and their affinity for neurotransmitters and drugs or inhibit ion pump operations thereby changing ion homeostasis. It has also been proposed that peroxidized membrane phospholipids in brain cells may produce a packing defect, making the sn-2 position more accessible to PLA<sub>2</sub> action (McLean et al., 1993). Several investigators have suggested that phospholipid hydroperoxides are better substrates for the activity of PLA<sub>2</sub> than the native lipid itself (McLean et al., 1993). The lipolysis of peroxidized lipids removes the oxidized fatty acyl chains, which are reduced and later re-incorporated. Thus PLA<sub>2</sub> action plays repairing and restorative roles in the maintenance of the physicochemical state of membranes.

PLA<sub>2</sub> and acyltransferase enzymes may participate in several key events that determine the turnover of phospholipids in cell membranes through the deacylation and reacylation cycle (Van den Bosch, 1980; Waite, 1987). This cycle is responsible for the introduction of PUFAs into phospholipids, which are also broken down by the phospholipase A<sub>2</sub> enzymes into lysophospholipids (LPLs). LPLs are re-esterified with free fatty acids to form other phospholipid molecules. The deacylation (hydrolytic) pathway is an important mechanism for the regulation of saturated and unsaturated fatty acyl contents of phospholipids in neural cell membranes (Yamashita et al., 1997). Among other various enzyme-regulating activities, LPLs serve as precursors of platelet activating factors (PAFs) (Weltzien, 1979).

AA incorporation into phospholipids is unique when compared with other fatty acids. First, the mechanism responsible for the esterification of AA in a variety of cell types appears to be highly selective for this fatty acid (Irvine, 1982). Second, the major pathway for AA incorporation into phospholipids is not the *de novo* pathway, but by the

deacylation/reacylation cycle (Lands and Crawford, 1976). It is thought that this cycle is largely responsible for the remodelling of cellular phospholipids leading to the selective distribution of AA at the sn-2 position which is cleaved by PLA<sub>2</sub> producing LPLs and this is rapidly re-esterified with another fatty acid by a CoA-dependent acyltransferase enzyme reforming a phospholipid (Hill, 1968; Dennis, 1987). AA uptake into phospholipids is dependent on the availability of LPLs particularly LPC. In P388D<sub>1</sub> macrophages (Balsinde et al., 1995) and human polymorphonuclear neutrophils, the levels of LPC appear to be maintained by iPLA<sub>2</sub> and not cPLA<sub>2</sub> activity (Daniele et al., 1999).

In schizophrenia, several reports have shown alterations in phospholipid metabolism (Pettegrew et al., 1991; Fujimoto et al., 1992). Perhaps, these observations can be explained by changes in the functioning of the LPL metabolising enzyme in the disorder. This study examined the esterification of AA into phospholipids after oxidation using U937 monocytic cells as models. Evidence is presented that a PLA<sub>2</sub> plays a role in this process by providing the LPL required for AA esterification into phospholipids. This study aims to provide more information on the enzymes involved in the metabolism of phospholipids, possibly allowing better understanding of their role in neuronal cell membranes.

## **7.2 MATERIALS AND METHODS**

### **7.2.1 Materials**

[5, 6, 8, 9, 11, 12, 14, 15-<sup>3</sup>H] AA (<sup>3</sup>H-AA, 100 Ci/mmol) and Optiphase Hisafe 2 liquid scintillation cocktail were purchased from Perkin-Elmer Life Sciences, UK. The fatty-acid-free bovine serum albumin (BSA), stearic acid (SA), oleic acid (OA), arachidonic acid (AA), phosphatidyl choline, ascorbic acid, vitamin E,  $\beta$ -carotene, astaxanthin,

0.4% Trypan Blue dye solution, Hanks balanced salt solution (HBSS), RPMI 1640, foetal bovine serum (FBS), 200 mM L-glutamine, 50 mg/ml gentamicin solution, HPLC-grade chloroform, hexane, diethyl ether, methanol and ethanol, glacial acetic acid, potassium chloride (KCl), iodine crystals, butylated hydroxy-toluene (BHT), tert butyl hydroperoxide, ferrous sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) and sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) were obtained from Sigma-Aldrich, UK. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were supplied by Nu-chek Prep. Inc., Denmark. The LK5D – Whatman TLC plates were from Fisher Scientific, UK.

### **7.2.2 Glassware and plastic preparations**

All glassware used was washed in de-con and nano-pure water, autoclaved at 121°C for 30 min and dried in an oven before use. All other apparatus used were sterile with all U937 cell treatments performed under a class II laminar flow hood. The laminar flow hood was cleaned regularly with 70% ethanol. Solvent extractions and thin layer chromatographic analysis were done in the designated area in the radiation suite.

### **7.2.3 Cell culture**

U937 cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine and 0.05 mg/ml gentamicin at 37°C in a humidified incubator with 5%  $\text{CO}_2$ /95% air. For all experiments, cells were maintained at  $0.5 \times 10^6$  cells/ml at viability > 90% by Trypan Blue dye exclusion method of viability measurements.

### **7.2.4 Cellular uptake of $^3\text{H}$ -AA into phospholipids**

#### ***7.2.4.1 Uptake under normal incubation conditions***

Unlabelled U937 cells were adjusted to contain  $0.5 \times 10^6$  cells/ml in 24-well plastic culture plates (Costar). Initially 0.9 ml of exogenous  $^3\text{H}$ -AA (0.1  $\mu\text{Ci/ml}$ ) in CM was

added to 0.1 ml of cell suspension for varying periods of time at 37°C and 5% CO<sub>2</sub> in a humid environment. The final volume per well was 1.0 ml. At the indicated times, cellular uptake was terminated by centrifuging at 4000 g for 3 min at 4°C followed by measurements of radioactivity remaining in the supernatant (7.2.7) and total <sup>3</sup>H-AA incorporated into phospholipids (7.2.8).

#### ***7.2.4.2 Oxidation of cells***

##### ***(A) Time dependent oxidation before uptake***

0.5 x 10<sup>6</sup> cells/ml of unlabelled U937 cells were washed twice in HBSS by centrifuging at 250 g for 5 min at 4°C to remove serum-containing CM and re-suspended in 0.1 ml of HBSS in each well of a 24-well plate. Stock solutions of 2 mM t-BHP and 5 mM FeSO<sub>4</sub> were prepared in HBSS just before use. Oxidative stress was induced by the addition of 0.9 ml of the oxidant mixture in HBSS (or HBSS only for controls) to 0.1 ml of cell suspension. Final volume was 1.0 ml/well. The final oxidant concentration used in all treatments was 750 μM t-BHP/Fe<sup>2+</sup>. The 0 h experiment (controls) involved cells in HBSS only for 4 h before the uptake assay. After varying oxidation times (0-4 h), cell suspensions were rapidly transferred into 1.5 ml eppendorf tubes and reactions were stopped by centrifuging at 250 g for 5 min at 4°C. Cells were washed twice with 0.9 ml HBSS, re-suspended in 0.1 ml of CM and labelled by adding 0.9 ml of <sup>3</sup>H-AA (0.1 μCi/ml) in CM. After 4 h of further incubation, <sup>3</sup>H-AA incorporation assay was terminated by centrifugation at 4000 g for 3 min at 4°C. Thereafter, uptake of <sup>3</sup>H-AA into cellular phospholipids was measured (7.2.8).

##### ***(B) Time-dependent uptake post-oxidation***

U937 cells adjusted to contain 0.5 x 10<sup>6</sup> cells/ml were initially treated/untreated with 750 μM t-BHP/Fe<sup>2+</sup> in HBSS (or HBSS only in a simultaneously conducted

experiment) in 24-well plates. Oxidant solutions containing t-BHP and FeSO<sub>4</sub> were freshly prepared just before use as previously stated. After 1 h of induced oxidation (or incubations in HBSS only), reactions were terminated by centrifugation at 250 g for 5 min at 4°C. Cells were washed twice each time with 0.9 ml HBSS and re-suspended in 0.1 ml of freshly prepared CM. Oxidant-treated/untreated cells (0.1 ml in CM) were labelled by the addition of 0.9 ml of <sup>3</sup>H-AA (0.1 μCi/ml) in CM for 0 – 24 h. The final volume of cell suspension in each well was 1.0 ml. After the indicated time, uptake reactions were terminated by centrifugation at 4000 g for 3 min at 4°C followed by the measurements of total cellular uptake of <sup>3</sup>H-AA into phospholipids (7.2.8).

## **7.2.5 With FA treatments**

### **7.2.5.1 Without oxidation**

#### **(A) Simultaneous addition of FA and <sup>3</sup>H-AA**

10 mM stock solutions of fatty acids (SA, OA, AA, EPA and DHA) were aseptically prepared in ethanol. They were used in U937 cell incubations complexed to fatty-acid-free BSA at a molar concentration of 2.5: 1. CM was supplemented with FA: BSA complexes and 0.1 μCi/ml of <sup>3</sup>H-AA to make a final volume of 0.9 ml, which was then added to 0.1 ml of cell suspension containing 0.5 x 10<sup>6</sup> cells/ml in each well of a 24-well plate. Controls involved cells incubated with ethanol (0.4% v/v) complexed with BSA only. The final FA concentration was 10 μM and the final volume was 1.0 ml/well. After 24 h of incubation at 37°C and 5% CO<sub>2</sub> in a humid environment, uptake reactions were terminated by centrifugation at 4000 g for 3 min at 4°C and <sup>3</sup>H-AA uptake into phospholipids was measured (7.2.8).



**(B) With unlabelled FA before  $^3\text{H-AA}$**

$0.5 \times 10^6$  cells/ml were initially incubated for 24 h with 10  $\mu\text{M}$  of the fatty acids: SA, OA, AA, EPA or DHA (or 0.4% v/v, ethanol for controls) complexed with BSA at 2.5:1 molar ratio. Afterwards, U937 cells were washed twice each time with 0.9 ml of HBSS/0.2% BSA by centrifugation at 250 g for 5 min at 4 °C and re-suspended in 0.1 ml of freshly prepared CM. FA-treated cells were then labelled with 0.9 ml of exogenous  $^3\text{H-AA}$  (0.1  $\mu\text{Ci/ml}$ ) in CM and incubated for a further 4 h under the conditions stated above. Final volume was 1.0 ml/well. Uptake reactions were stopped by centrifugation at 4000 g for 3min at 4°C followed by measurements of radioactivity incorporated into cellular phospholipids (7.2.8).

**7.2.5.2 With Oxidation**

Furthermore,  $0.5 \times 10^6$  cells/ml were cultured in CM supplemented with 10  $\mu\text{M}$  SA, OA, AA, EPA or DHA (or 0.4% v/v, ethanol for controls) at 37°C and 5%  $\text{CO}_2$  in a humid environment. After 24 h of incubation, cells were washed twice with 0.9 ml of HBSS/0.2% BSA by centrifugation at 250 g for 5 min at 4°C and re-suspended in 0.1 ml of HBSS. U937 cells were then treated with/without 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  (freshly prepared in HBSS from 2 mM t-BHP and 5 mM  $\text{FeSO}_4$  just before use) and incubated under the conditions stated above. After 1 h, oxidant-treated cells were washed twice with 0.9 ml of HBSS and re-suspended in 0.1 ml of CM in each well of a 24-well plate prior to labelling with 0.9 ml of  $^3\text{H-AA}$  (0.1  $\mu\text{Ci/ml}$ ) in CM for a further 4 h.  $^3\text{H-AA}$  uptake reactions were stopped by centrifugation at 4000 g for 3 min at 4°C. Cellular uptake of  $^3\text{H-AA}$  into phospholipids was measured (7.2.8).

Controls (Ox and – Ox) involved cells treated with ethanol (0.4% v/v) complexed with BSA and incubated with and without 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ , respectively.

### 7.2.5.3 With prolonged FA treatment

U937 cells were continuously cultured for 18 days in CM supplemented with 10  $\mu\text{M}$  levels (final concentration) of the FAs (SA, OA, AA, EPA or DHA) at 37°C and 5%  $\text{CO}_2$  in a humid environment. All FAs were complexed with BSA in a 2.5: 1 molar ratio. For controls, cells were incubated with 0.4% (v/v) ethanol (final volume) complexed with BSA. Cells were cultured in 8 ml cell suspensions at  $0.5 \times 10^6$  cells/ml in 25  $\text{cm}^2$  culture flasks. The incubation medium was totally renewed with freshly prepared CM supplemented with 10  $\mu\text{M}$  of the FA complex every 3 days. On day 17, U937 cells were again re-adjusted to contain  $0.5 \times 10^6$  cells/ml and re-suspended in 1 ml suspensions in 24-well plates for a further 24 h in freshly prepared CM. After a total of 18 days, cells were washed twice in 0.9 ml HBSS/0.2% BSA by centrifugation at 250 g for 5 min at 4°C, re-suspended in 0.1 ml of HBSS and subjected to oxidative stress for 1 h with 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  (prepared in HBSS from 2 mM t-BHP and 5 mM  $\text{FeSO}_4$  just before use). Oxidation reactions were terminated by centrifugation at 250 g for 5 min at 4°C. Cells were washed twice with 0.9 ml of HBSS, re-suspended in 0.1 ml of freshly prepared CM and labelled with 0.9 ml of exogenous  $^3\text{H}$ -AA (0.1  $\mu\text{Ci/ml}$ ) in CM for 4 h. Final volume of cell suspension per well was 1.0 ml. Thereafter, radioactivity of the total uptake of  $^3\text{H}$ -AA into cellular phospholipids was measured (7.2.8).

Controls (Ox and – Ox) involved U937 cells treated with ethanol (0.4% v/v) complexed with BSA and incubated with and without 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ , respectively. Cell viability and cell density was monitored every 3 days and maintained at  $0.5 \times 10^6$  cells/ml at a viability > 90% by the Trypan Blue dye-exclusion method. Cell suspensions remaining after cell counts were discarded and treated appropriately.

### 7.2.6 With antioxidants

100 mM stock solutions of ascorbic acid in water,  $\alpha$ -tocopherol in ethanol,  $\beta$ -carotene and astaxanthin in chloroform were aseptically prepared. Ascorbic acid and  $\alpha$ -tocopherol were stored at 2 – 8°C, while  $\beta$ -carotene and astaxanthin were stored at – 20°C. By serial dilution, 16 mM ascorbic acid, 40 mM  $\alpha$ -tocopherol and 12 mM  $\beta$ -carotene and astaxanthin were prepared. Culture medium (CM) was supplemented with aliquots of 16 mM ascorbic acid, 40 mM  $\alpha$ -tocopherol and 12 mM  $\beta$ -carotene or astaxanthin, all to a final volume of 0.8 ml. Antioxidant supplemented CM (0.8 ml), was added to each of a 24 – well plate containing 0.1 ml of cell suspension after appropriate treatments (in the case of the carotenoids where, 0.8 ml of carotenoid – supplemented CM was sonicated for 5 s in an ice-bath and sterile – filtered using nylon syringe filters before use). Each well contained  $0.5 \times 10^6$  cells/ml. Final concentrations of antioxidants used were 400  $\mu$ M ascorbic acid, 40  $\mu$ M  $\alpha$ -tocopherol and 12  $\mu$ M  $\beta$ -carotene or astaxanthin. U937 cells were then incubated for an initial 10 min with each of the antioxidants before the addition of 0.1 ml of PUFA: BSA complexes (2.5: 1 molar ratio) in CM. The final PUFA (AA, EPA or DHA) concentration was 10  $\mu$ M in 1.0 ml cell suspensions. After 24 h of incubation, cells were washed twice with HBSS/0.2% BSA and re-suspended in 0.1 ml of HBSS followed by oxidation with 750  $\mu$ M t-BHP/ $\text{Fe}^{2+}$  for 1 h. Post-oxidation, cells were washed twice with 0.9 ml of HBSS and re-suspended in 0.1 ml of CM in each well of a 24-well plate. Thereafter, oxidant-treated cells were labelled with 0.9 ml of exogenous  $^3\text{H}$ -AA (0.1  $\mu\text{Ci/ml}$ ) in CM for 4 h under the conditions stated above. Blank experiments (– FA, – Ox) were U937 cells treated with ethanol (0.4% v/v) complexed with BSA without the induction of oxidative stress. Controls (Ox and – Ox) involved cells treated with 10  $\mu$ M PUFA (AA, EPA or DHA) complexed with BSA in a 2.5: 1 molar ratio and treated with and without 750  $\mu$ M t-BHP/ $\text{Fe}^{2+}$ , respectively.

Uptake reactions were terminated by centrifugation at 4000 g for 3 min at 4°C followed by measurements of radioactivity taken up into cellular phospholipids (7.2.8).

All incubations were performed at 37°C and 5% CO<sub>2</sub> in a humid environment. All washings were performed at 250 g for 5 min at 4°C and all terminations prior to the measurements of radioactivity achieved by centrifugation at 4000 g for 3 min at 4°C.

#### **7.2.7 Measurement of radioactivity remaining in the supernatant**

0.9 ml of supernatants were removed and transferred into labelled 20 ml scintillation vials. Cell pellets were washed once with 0.9 ml of HBSS/BSA and 0.9 ml of the supernatant was carefully removed by aspiration and combined with the previous supernatant. 12 ml of scintillation fluid was added to the combined supernatant, mixed by vortexing and assayed for radioactivity by liquid scintillation counting. Radioactivity was expressed as a percentage of the total radioactivity originally present in the medium.

#### **7.2.8 Measurement of cellular uptake of <sup>3</sup>H-AA into phospholipids**

After final centrifugation, cell suspensions were washed once with HBSS/0.2% BSA and re-suspended in 0.1 ml of HBSS/0.2% BSA. For the analysis of <sup>3</sup>H-AA incorporated into phospholipids, 0.8 ml (8 volumes) of ice-cold, chloroform: methanol (2: 1, v/v) (Folch et al., 1957) was added to 0.1 ml of cell suspension. Cells were sonicated for 3 x 10 s with an interval of 10 s in an ice bath followed by the rapid addition of 0.2 ml (0.2 volumes) of ice-cold methanol. Suspensions were mixed briefly by vortexing and centrifuged at 4000 g for 3 min at 4°C. Centrifugation was used to convert all non-lipid residues into compact pellets instead of filtration due to the small sample size. 0.7 ml of supernatant was transferred into labelled Pyrex tubes and 0.2 ml

(25% of the existing volume) of 0.88%KCl solution was added. Tubes were well shaken and allowed a few minutes for separation into two phases. Aliquots of 0.2 ml of the bottom layer were transferred into sterile vials and evaporated to dryness under a stream of nitrogen. Cellular extracts were re-suspended in 30  $\mu$ l of 3.33 mM exogenous PC in chloroform: methanol (2: 1, v/v with 0.01% BHT) in airtight vials and stored for a maximum of 72 h at  $-20^{\circ}\text{C}$  prior to TLC analysis of  $^3\text{H}$ -AA uptake.

### 7.2.9 Thin layer chromatographic separations

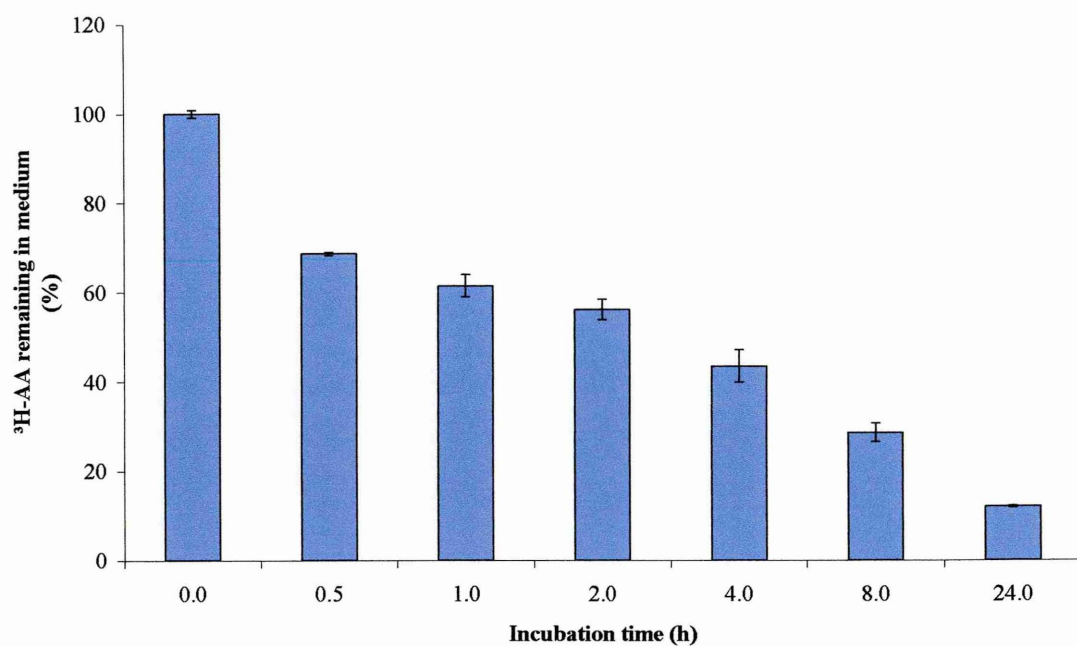
A solvent system of hexane: diethyl ether: acetic acid, 70: 30: 1.3 (v/v/v) was made up and stored in Schott bottles. Using a micro-Pasteur pipette, 10 - 20  $\mu$ l aliquots of lipid extracts were spotted on LK5D plates under a stream of nitrogen gas with reference phosphatidyl choline (PC) co-chromatographed as standards on separate lanes for identification purposes. The plates were run in a chamber pre-saturated with 100 ml of the solvent system above. After 50 – 55 min, plates were dried with cool air from a hair dryer for  $\sim 30$  s in a fume cupboard until free of the acetic acid odour. Thereafter, plates were exposed to iodine vapour produced from iodine crystals in a closed tank. Lipid spots corresponding to standard phospholipid bands (for total AA incorporation) were outlined with a needle (PL spots did not migrate from the origin). TLC plates were removed from the tank and iodine was allowed to sublime. Spots ( $\leq 2$  cm $^2$ ) were scraped with a scalpel and transferred via a small plastic funnel into 7 ml scintillation vials containing 0.4 ml of 1%  $\text{Na}_2\text{S}_2\text{O}_3$ , in order to reduce the iodine to iodide ion, thus decolorizing the mixture and minimizing quenching (as modified by Horrocks and Ansell, 1967). 4.0 ml of optiphase hisafe 2 liquid scintillation cocktail was added, mixed vigorously by vortexing and radioactivity was quantified by liquid scintillation spectroscopy in disintegrations per minute using the 1219 Rackbeta liquid scintillation counter by LKB Wallac.

$^3\text{H}$ -AA incorporation into cellular phospholipids was expressed as a percentage of the total radioactivity originally present in the medium.

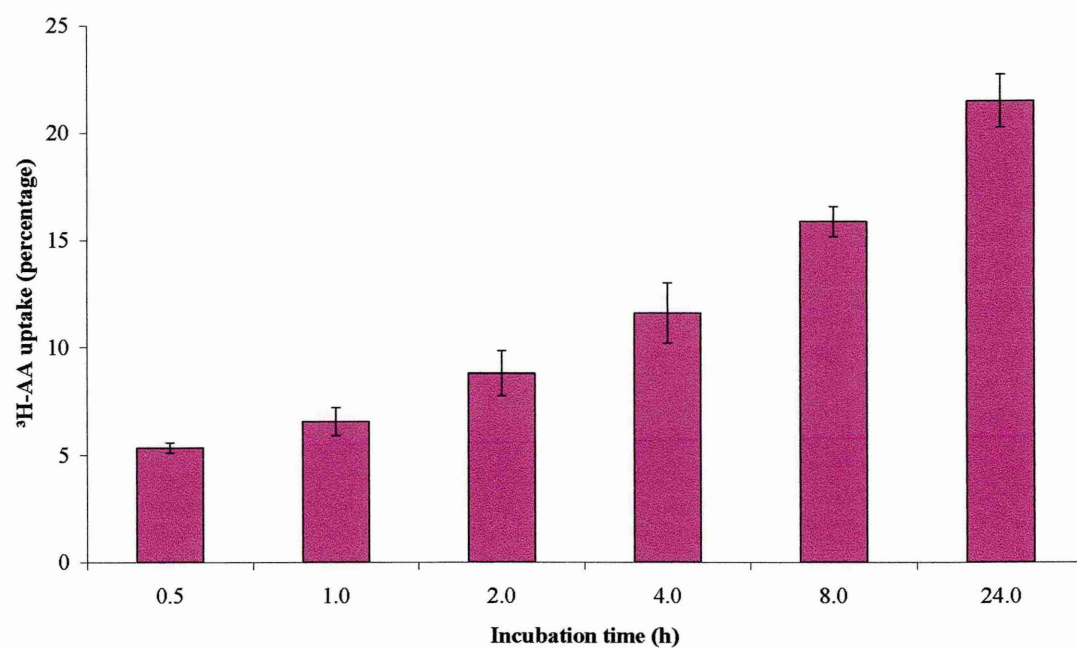
### 7.3 RESULTS

Initial experiments were performed to determine the extent of incorporation of exogenous, radiolabelled AA into U937 cell phospholipids over time (0 – 24 h). As shown in Fig. 7.1A,  $^3\text{H}$ -AA was rapidly taken up by the cells with approximately 69% of the total  $^3\text{H}$ -AA added to the medium remaining in the supernatant after the first 30 min of incubation suggesting that 31% of the total radioactivity was cell-associated in this time. There is a good negative correlation between  $^3\text{H}$ -AA content remaining in the medium and time ( $R^2 = -0.97$ ,  $P < 0.01$ ). Approximately 40% of the total radioactivity added to the cells was left in the incubation medium after 4 h of incubation and 10% after 24 h. The same pattern of  $^3\text{H}$ -AA uptake was observed in cell phospholipids after thin layer chromatographic analysis of lipid extracts (Fig. 7.1B) where the amount esterified into phospholipids increased linearly with incubation time ( $R^2 = 0.977$ ,  $P < 0.01$ ). There was an increase of 120% and about 300 % in  $^3\text{H}$ -AA incorporation into phospholipids at 4 and 24 h (respectively) of incubation when compared to cellular uptake at 30 min of incubation.

The rate of incorporation of radioactivity into cell phospholipids was greatest within the first 30 min of incubation and this reduced with time. The average rate per hour of  $^3\text{H}$ -AA uptake into phospholipids was approximately 97% greater at 30 min of incubation than that obtained at 24 h (Fig. 7.1B). This study demonstrates that the incorporation of  $^3\text{H}$ -AA into phospholipids continues for 24 h in U937 cells with approximately 10% of the radiolabelled AA remaining in the medium after this time (Fig. 7.1A).



(A)



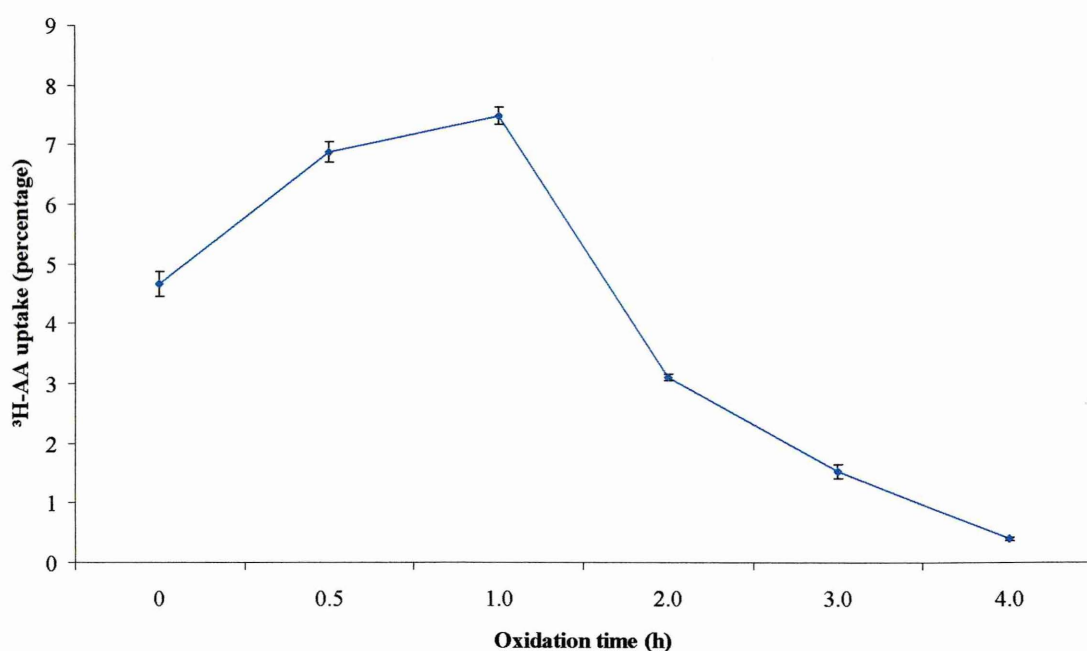
(B)

**Fig. 7.1** <sup>3</sup>H-AA incorporation into U937 cell phospholipids. Cells were incubated for 0.5 - 24 h and the total amount of AA remaining in the medium (A) or aliquots of <sup>3</sup>H-AA incorporated into phospholipids (B), was measured. Both were expressed as a percentage of total radioactivity originally added to the medium. Data points represent means  $\pm$  95% CIs of two independent experiments (n = 4).

Whilst there was no significant increase in uptake between 0.5 and 1.0 h of incubation, it increased significantly at each of the other time points indicated over 1 h (one-way ANOVA  $F = 154.93$ ,  $P < 0.01$ ,  $DF = 5$ ).

### 7.3.1 Time dependent oxidation pre- $^3\text{H}$ -AA uptake

Given the ability of the U937 cells to rapidly incorporate  $^3\text{H}$ -AA into phospholipids, the effects of oxidation on its uptake were investigated (Fig. 7.2).



**Fig. 7.2** Effect of U937 cell treatments with  $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for varying periods of time (0 – 4 h) on the esterification of  $^3\text{H}$ -AA into phospholipids. Means  $\pm$  95% CIs of duplicate experiments ( $n = 4$ ).

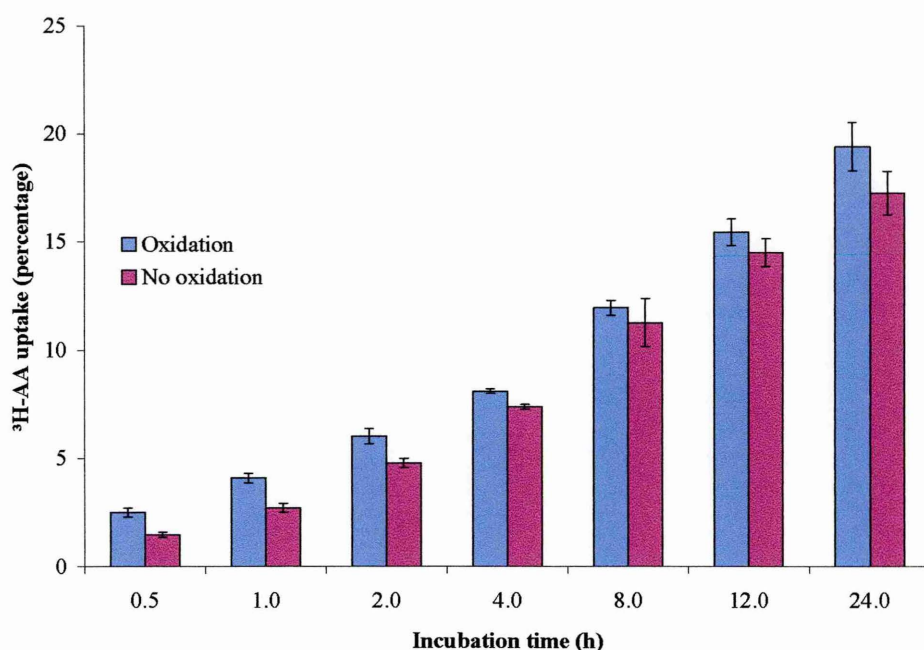
To demonstrate the oxidant-stimulated turnover of membrane phospholipids, U937 cells were initially treated with  $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for 0 – 4 h in HBSS, washed twice to ensure the removal of t-BHP/ $\text{Fe}^{2+}$  remaining in the medium, before the addition of  $0.1\ \mu\text{Ci/ml}$  of  $^3\text{H}$ -AA in freshly prepared CM. Based on the substantial uptake of  $^3\text{H}$ -AA



into cellular phospholipids after 4 h of incubation (Fig. 7.1), incorporation assay proceeded for 4 h under the conditions employed (Fig. 7.2). Interestingly, there was a significant increase in  $^3\text{H}$ -AA uptake into phospholipids after 30 min of oxidant treatment of U937 cells. The increase peaked at 1 h of oxidation after which there was a dramatic decrease in cellular uptake for the following 3 h (one-way ANOVA  $F = 2166.70$ ,  $P < 0.01$ ,  $DF = 5$ ). This data demonstrates that the chronic exposure of U937 cells to  $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for  $> 1$  h alters the acyltransferase enzyme in some way, dramatically reversing the uptake response initially observed with oxidation times  $\leq 1$  h. This may be due to increasing loss of membrane integrity over time as shown earlier in chapter two. Interestingly, AA uptake at oxidation times  $> 1$  h, coincides with results obtained with increasing AA release experiments over time (chapter six), so it could be that induced-oxidative stress with time stimulates AA release at a faster rate than its uptake in U937 cells. Based on this study, all subsequent oxidation reactions were performed with  $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for 1 h prior to  $^3\text{H}$ -AA uptake assays.

### **7.3.2 Oxidant-induced $^3\text{H}$ -AA uptake into phospholipids**

Fig. 7.3 shows the time-course of t-BHP/ $\text{Fe}^{2+}$ -induced  $^3\text{H}$ -AA uptake into U937 cell phospholipids.



**Fig. 7.3** Time course of  $^3\text{H}$ -AA uptake into U937 cells pre-treated with or without  $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for 1 h in HBSS. Means  $\pm$  95% CIs ( $n = 4$ ).

Data points in Fig. 7.3 were expressed as percentages of radioactivity incorporated into phospholipids compared to the total radioactivity originally added to the medium. AA incorporation into cellular phospholipids increased over time from 0.5 to 24 h in both oxidant-treated and untreated U937 cells.

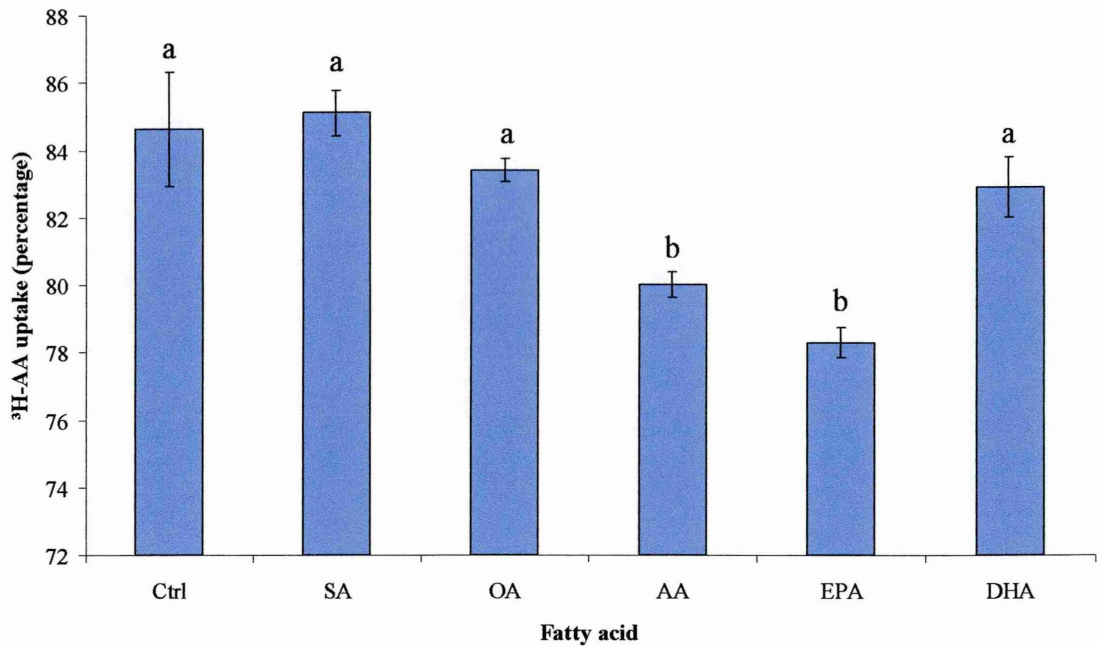
Fig. 7.3 shows that  $^3\text{H}$ -AA uptake increases significantly with increasing incubation time (one-way ANOVA  $F = 1261.65$ ,  $P < 0.001$ ,  $DF = 6$ ). It also demonstrates a significant difference in  $^3\text{H}$ -AA uptake between U937 cells treated with and without  $750\ \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for 1 h (one-way ANOVA  $F = 103.54$ ,  $P < 0.001$ ,  $DF = 1$ ). This data indicate that  $^3\text{H}$ -AA incorporation into phospholipids is greater in U937 cells with induced-oxidation compared to cells untreated with the oxidant, suggesting an increase in the activity of the acyltransferase enzyme with oxidative stress.

**7.3.3 With FA treatments**

The effect of different fatty acids on <sup>3</sup>H-AA incorporation into phospholipids was investigated under different circumstances.

**7.3.3.1 Without oxidation**

(A) The first condition employed involved the exposure of U937 cells to either 10 μM SA, OA, AA, EPA, DHA or ethanol (controls) concomitantly with <sup>3</sup>H-AA (0.1 μCi/ml) in CM for 24 h (Fig. 7.4).

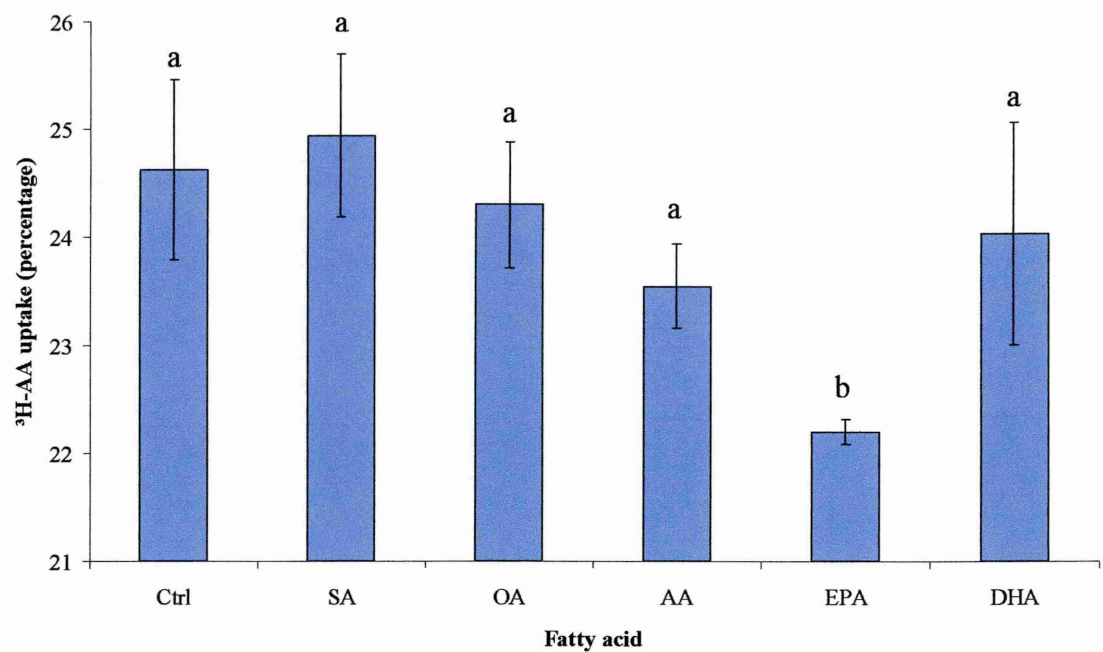


**Fig. 7.4** Effect of the simultaneous addition of ethanol (controls) or the fatty acids (SA, OA, AA, EPA or DHA) and <sup>3</sup>H-AA on uptake into phospholipids with measurements performed after 24 h of incubation. Means ± 95% CIs (n = 4). Letters: a and b denote significant differences between treatments (P < 0.01).

As illustrated in Fig. 7.4, <sup>3</sup>H-AA incorporation was neither affected by co-treatment with SA nor OA for 24 h but was significantly impaired by the presence of the 20-

carbon PUFAs (AA and EPA) when compared to controls (one-way ANOVA  $F = 37.31$ ,  $P < 0.01$ ,  $DF = 5$ ). Interestingly, the uptake of  $^3\text{H}$ -AA was not significantly affected by the presence of DHA in the incubation medium. EPA-, though not significantly different from AA-treated cells gave the lowest observed uptake of  $^3\text{H}$ -AA by approximately 8% when compared to controls.

(B) Secondly, experiments examining the effects of incubating U937 cells with 10  $\mu\text{M}$  concentrations of the different fatty acids for 24 h before the uptake assay with  $^3\text{H}$ -AA for 4 more hours were conducted. Cells were washed twice with HBSS/0.2%BSA to eliminate all unlabelled and unincorporated fatty acids previously added to the culture medium (Fig. 7.5).

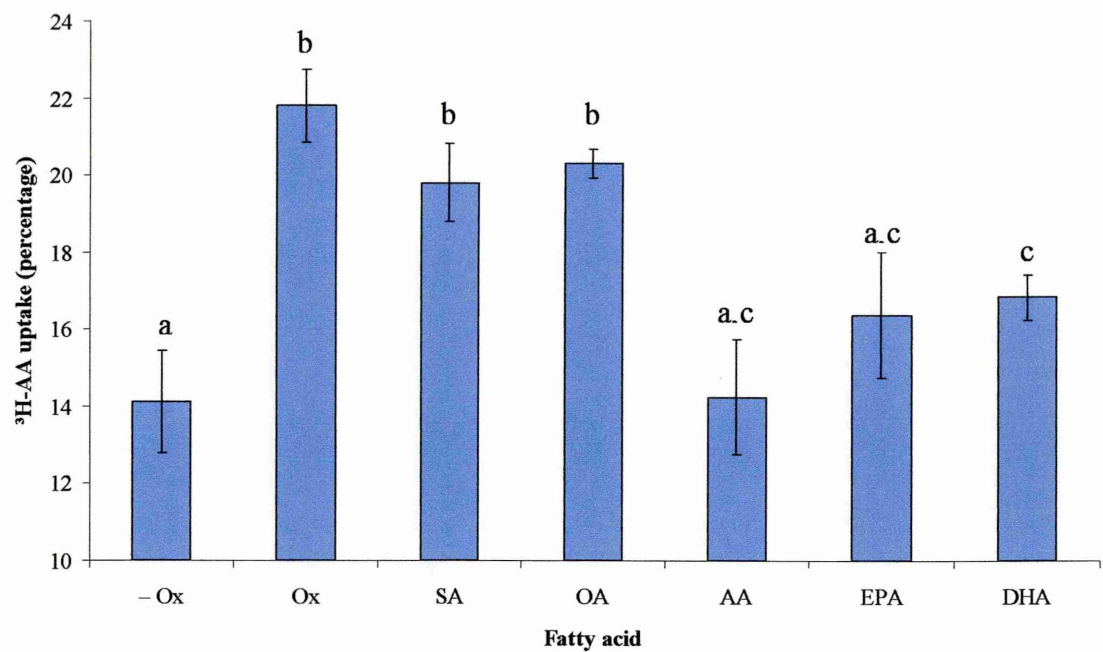


**Fig. 7.5**  $^3\text{H}$ -AA incorporation into phospholipids (for 4 h) after previous treatments of U937 cells with unlabelled SA, OA, AA, EPA or DHA for 24 h. Values represent means  $\pm$  95% CIs ( $n = 4$ ). Letters: a and b indicate significant differences between treatments ( $P < 0.01$ ).

After 4 h of incubating FA-treated cells with  $^3\text{H}$ -AA, there was no significant difference in uptake with SA, OA, AA or DHA. However, uptake was significantly inhibited by the presence of EPA in U937 cells (one-way ANOVA  $F = 8.06$ ,  $P < 0.01$ ,  $DF = 5$ ). The uptake assay with  $^3\text{H}$ -AA was conducted for 4 h because it was previously established that a high amount of  $^3\text{H}$ -AA was incorporated into phospholipids at that time (Fig. 7.1).

**7.3.3.2 With oxidation**

In order to determine the effects of induced – oxidative stress on  $^3\text{H}$ -AA uptake in FA-treated cells, U937 cells were pre-treated for 24 h and challenged with 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  for 1 h before the uptake assay with labelled AA was performed for 4 h (Fig. 7.6).



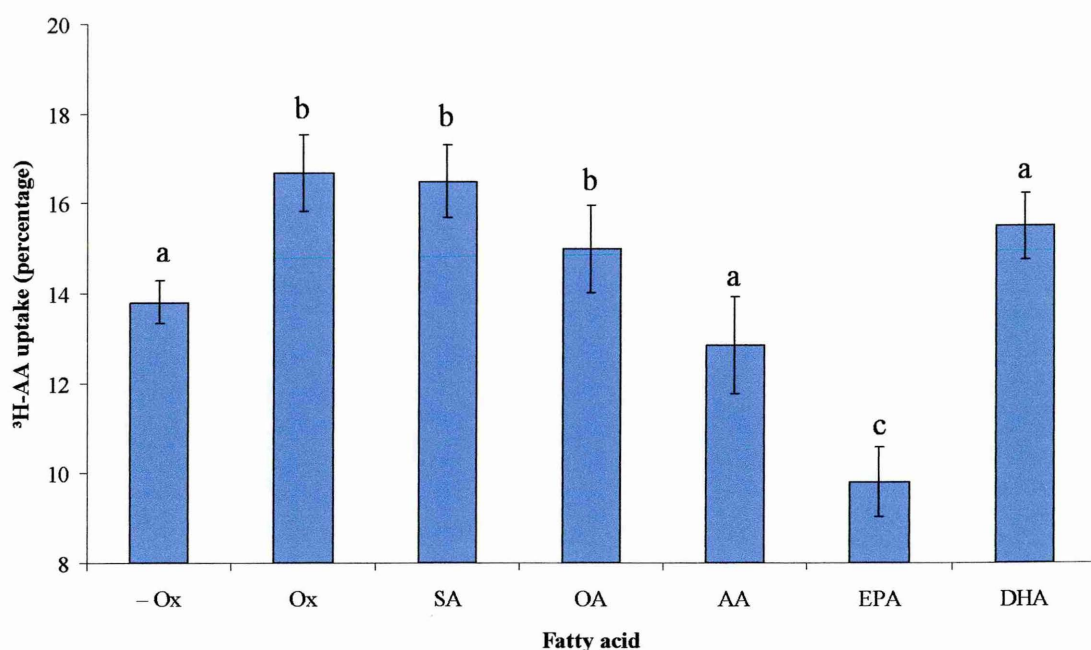
**Fig. 7.6** Effect of oxidation on  $^3\text{H}$ -AA incorporation (for 4 h) in FA – pre-treated U937 cells. Means  $\pm$  95% CIs ( $n = 4$ ). Letters: a, b and c denote significant differences between means ( $P < 0.01$ ).

After induced oxidation, FA-treated U937 cells were exposed to 0.1  $\mu\text{Ci/ml}$  of  $^3\text{H-AA}$  for 4 h prior to lipid extraction and TLC analysis. As expected, comparison between the controls confirms that  $^3\text{H-AA}$  uptake markedly increases in U937 cells with oxidation (one-way ANOVA  $F = 22.85$ ,  $P < 0.01$ ,  $DF = 6$ ). Pre-treatment with SA or OA showed no significant difference when compared with oxidized controls (Ox). However, pre-incubations with the PUFAs (AA, EPA or DHA) demonstrate a significant reduction in the uptake of  $^3\text{H-AA}$  compared to oxidized controls (Ox). Whilst uptake of  $^3\text{H-AA}$  was significantly different in DHA-pre-treated cells compared to unoxidized controls ( $- \text{Ox}$ ), there were no significant differences observed with AA or EPA treatments suggesting that AA and EPA presence suppressed the uptake of  $^3\text{H-AA}$  in U937 cells subjected to oxidative stress in a similar manner to controls untreated with oxidants ( $- \text{Ox}$ ) (Fig. 7.6).

#### **7.3.4 With prolonged FA treatment**

To further substantiate the above findings, experiments utilizing U937 cells continuously pre-cultured in CM-supplemented with 10  $\mu\text{M}$  levels of the five different fatty acids were subjected to oxidation for 1 h prior to the conduction of cellular  $^3\text{H-AA}$  incorporation for 4 h (Fig. 7.7).





**Fig. 7.7** Incorporation of <sup>3</sup>H-AA into cellular phospholipids after 18 days of continuous CM-supplementation with SA, OA, AA, EPA, DHA or ethanol (controls) followed by 1 h of induced oxidation. Data points represent means  $\pm$  95% CIs (n = 4). Letters: a, b and c indicate significant differences between treatments ( $P < 0.01$ ).

Prolonged FA-treatment of cells generated more pronounced effects of the PUFAs especially EPA, in response to oxidant-induced <sup>3</sup>H-AA uptake into U937 cell phospholipids. Again, the oxidation of U937 cells resulted in increased <sup>3</sup>H-AA uptake in oxidant-treated cells (Ox) compared to unoxidized controls. <sup>3</sup>H-AA incorporation was not affected by prolonged treatment with the saturated or monounsaturated fatty acids (SA or OA respectively) when compared to oxidant-treated controls (one-way ANOVA  $F = 33.53$ ,  $P < 0.01$ ,  $DF = 6$ ). Comparison of the PUFA-treated cells showed that the presence of DHA resulted in the highest observed <sup>3</sup>H-AA uptake into cellular phospholipids. EPA-treatment showed the most suppressive effects on AA-uptake by approximately 40% lower than oxidized (Ox) controls. The suppressive effect was greater with EPA than AA-treatments. Whilst <sup>3</sup>H-AA uptake was significantly lower in

AA and EPA treated cells compared to oxidized controls, pre-incubation with DHA showed no significant differences. In comparison to oxidant-untreated (– Ox) controls,  $^3\text{H}$ -AA uptake was markedly lower with EPA- whereas AA-pre-treated U937 cells were not.

Surprisingly, the data represented in Fig. 7.7 ( $^3\text{H}$ -AA uptake after FA pre-treatment for 18 days) looks strikingly more similar to that in Fig. 7.5 (without induced-oxidation) than Fig. 7.6 (with induced-oxidation), suggesting pronounced effects of the fatty acids alone and not that induced by pro-oxidants. Unfortunately this suggestion cannot be confirmed in the present study because control experiments did not include cells treated with the different fatty acids for 18 days without exposure to the oxidants. Future work is therefore required to investigate this possibility.

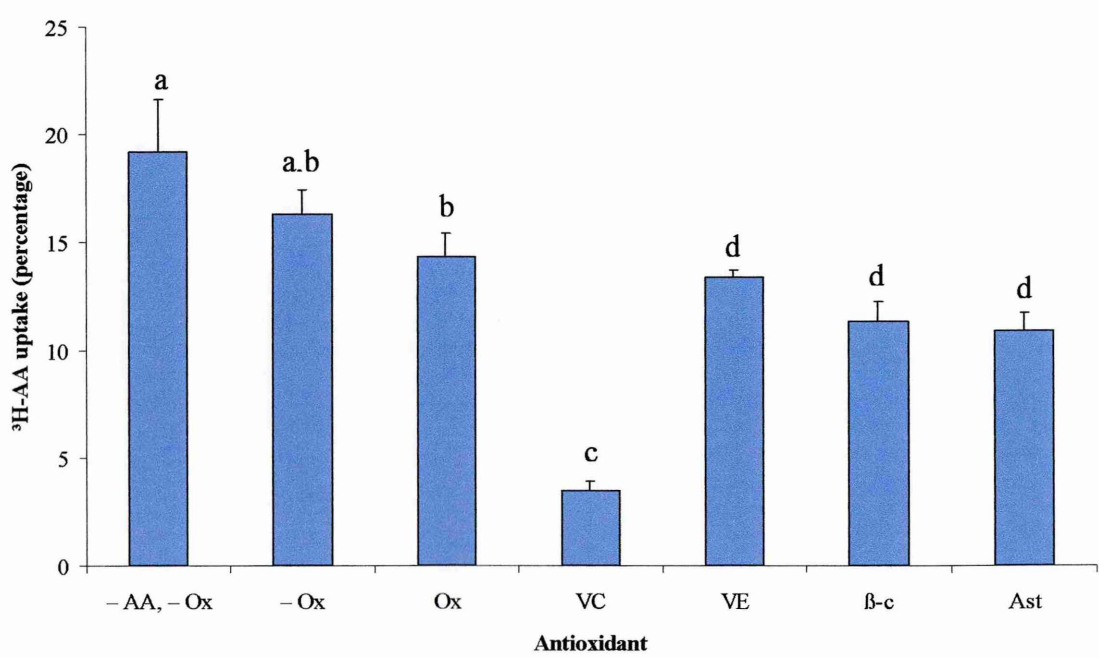
### 7.3.5 With Antioxidants

To define the role of ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin on  $^3\text{H}$ -AA incorporation into cellular phospholipids, U937 cells were pre-treated with 400  $\mu\text{M}$  ascorbic acid, 40  $\mu\text{M}$   $\alpha$ -tocopherol, 12  $\mu\text{M}$   $\beta$ -carotene or 12  $\mu\text{M}$  astaxanthin in the presence of 10  $\mu\text{M}$  AA, EPA or DHA for 24 h before the oxidant-stimulated  $^3\text{H}$ -AA uptake assay for 4 h was conducted. Fig. 7.8 shows the effects of the different antioxidants and PUFA treatments on oxidant-induced  $^3\text{H}$ -AA uptake into the phospholipids of U937 cells.

With AA treatment, there was no significant difference between U937 cells treated with (– Ox) and without (– AA, – Ox) 10  $\mu\text{M}$  AA, both without oxidation (one-way ANOVA  $F = 102.29$ ,  $P < 0.01$ ,  $DF = 6$ ). However, uptake of  $^3\text{H}$ -AA into cellular phospholipids in the blank treatment (– AA, – Ox) was significantly different from all treatments



involving oxidation. As observed earlier, no significant difference was observed between unoxidized (– Ox) and oxidized (Ox) controls, suggesting that U937 cell pre-treatment with unlabelled AA stimulated no significant difference in  $^3\text{H}$ -AA uptake with induced oxidative stress.

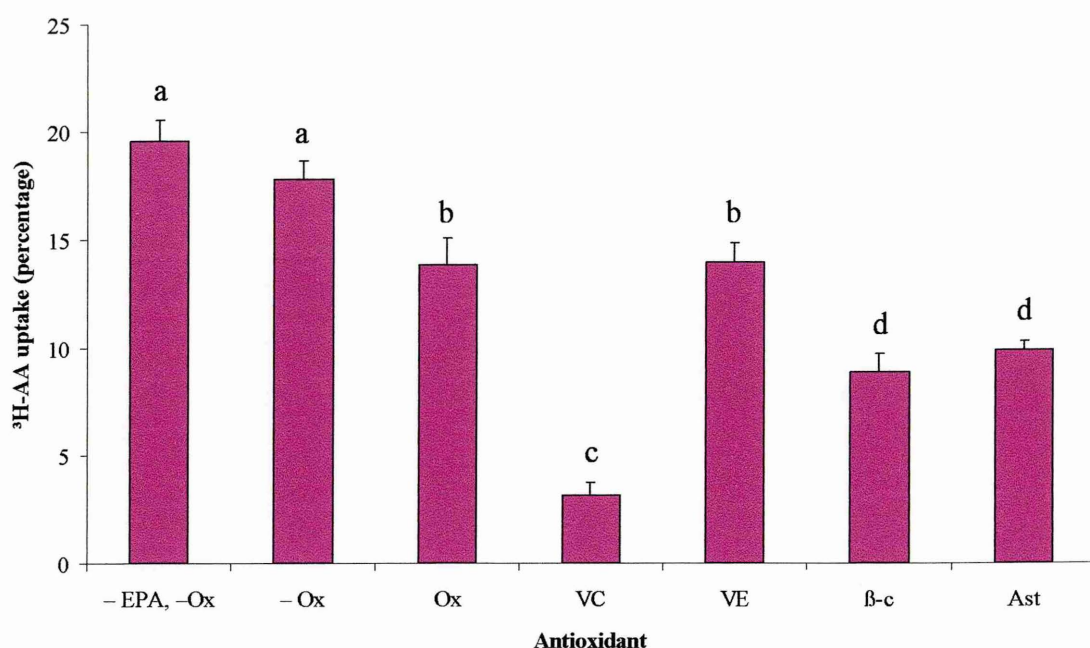


**Fig. 7.8** Effect of antioxidants and 10  $\mu\text{M}$  AA on oxidant-induced  $^3\text{H}$ -AA uptake into the phospholipids of U937 cells. Where: (– AA, – Ox) represents cells treated neither with AA nor oxidants; – Ox: control cells treated with AA without oxidants; Ox: cells treated with AA and oxidants; VC: AA + 400  $\mu\text{M}$  ascorbic acid; VE: AA + 40  $\mu\text{M}$   $\alpha$ -tocopherol;  $\beta$ -c: AA + 12  $\mu\text{M}$   $\beta$ -carotene; and Ast: AA + 12  $\mu\text{M}$  astaxanthin. All treatments with antioxidants were exposed to the oxidants. Means  $\pm$  95% CIs ( $n = 4$ ). Letters: a, b, c and d represent significant differences between treatments ( $P < 0.01$ ).

Unoxidized controls (– Ox) generated results that were significantly different from all treatments involving all the four different antioxidants. Although no significant

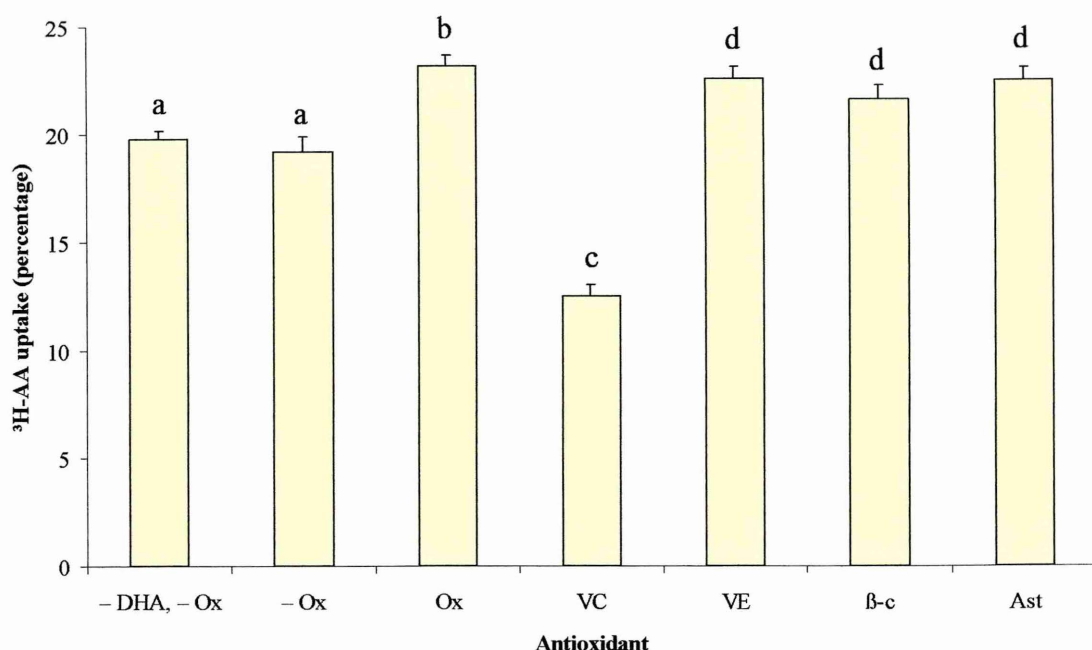
differences were observed between cells pre-treated with the three lipid-soluble antioxidants ( $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin), when compared with oxidant-treated controls (Ox), while  $\alpha$ -tocopherol did not exert any effect on  $^3\text{H-AA}$  incorporation into cellular phospholipids, treatments with  $\beta$ -carotene or astaxanthin resulted in significant but slight inhibition ( $P < 0.05$ ) of  $^3\text{H-AA}$  uptake into cellular phospholipids. However, treatment with 400  $\mu\text{M}$  ascorbic acid led to a dramatically significant reduction ( $P < 0.0001$ ) in the uptake of  $^3\text{H-AA}$  after 1 h of induced oxidation.

The effect of antioxidants and 10  $\mu\text{M}$  EPA treatments on  $^3\text{H-AA}$  incorporation into U937 cell phospholipids is shown in Fig 7.9. As previously shown in Fig. 7.6, there was a significant decrease in  $^3\text{H-AA}$  uptake between EPA treated cells with and without induced-oxidation (one-way ANOVA  $F = 181.91$ ,  $P < 0.01$ ,  $DF = 6$ ). Similar to results obtained with AA (Fig. 7.8), this study showed no significant differences in uptake between cells treated neither with 10  $\mu\text{M}$  EPA nor oxidants (– EPA, – Ox) and unoxidized controls.



**Fig. 7.9** Effect of four individual antioxidants and 10  $\mu$ M EPA on oxidant-induced  $^3\text{H}$ -AA uptake into the phospholipids of U937 cells. Means  $\pm$  95% CIs ( $n = 4$ ). Data in Fig 7.9 show that  $^3\text{H}$ -AA uptake in stimulated EPA-treated U937 cell phospholipids were markedly suppressed ( $P < 0.005$ ) with oxidation. Treatment with  $\alpha$ -tocopherol appeared to have no effect on U937 cells when compared with oxidized controls (Ox) whilst the presence of 12  $\mu$ M  $\beta$ -carotene and astaxanthin suppressed the uptake of AA. Ascorbic acid greatly reduced  $^3\text{H}$ -AA uptake by 77% compared to oxidant-treated controls (Ox). Letters: a, b, c and d denote significant differences between treatments ( $P < 0.01$ ).

The effect of treating U937 cells with 10  $\mu$ M DHA and antioxidants on oxidant-mediated  $^3\text{H}$ -AA incorporation into cellular phospholipids were also investigated (Fig. 7.10).



**Fig. 7.10** Effect of DHA (10  $\mu$ M) and four different antioxidants on the incorporation of  $^3\text{H-AA}$  in U937 cell phospholipids. Means  $\pm$  95% CIs ( $n = 4$ ). Letters: a, b, c and d indicate significant differences between treatments ( $P < 0.01$ ).

This study examined whether treatment of U937 cells with or without antioxidants and DHA promote oxidant-induced  $^3\text{H-AA}$  uptake in U937 cells (Fig. 7.10). As previously reported, pre-incubation with DHA for 24 h showed no significant differences between cells treated with and without 10  $\mu$ M DHA under oxidant-free conditions. In contrast to the response observed with AA or EPA treatments, induced-oxidation in DHA – treated cells generated a significant increase in  $^3\text{H-AA}$  uptake into cellular phospholipids (one-way ANOVA  $F = 181.88$ ,  $P < 0.01$ ,  $DF = 6$ ). In a similar manner to results obtained with AA or EPA treatments, 400  $\mu$ M ascorbic acid concentrations significantly reduced  $^3\text{H-AA}$  uptake into cellular phospholipids of DHA-treated cells. Collectively, there were no significant differences in  $^3\text{H-AA}$  observed in U937 cells pre-treated with either of the lipid-soluble antioxidants ( $\alpha$ -tocopherol,  $\beta$ -carotene or astaxanthin) when compared to controls.

## 7.4 DISCUSSION AND CONCLUSION

Much work has been reported examining the role of oxidative stress in the release of arachidonic acid (AA) in schizophrenia but little has been done to study the effect of oxidation on AA incorporation into phospholipids. The primary aim of this work was therefore to determine the effects of induced-oxidation on AA uptake into the phospholipids of U937 cells.

This study was important for determining whether PLA<sub>2</sub> in oxidant-stimulated U937 cells alters the endogenous AA pools in cellular phospholipids. For this purpose, experiments where the radiolabelled AA was added after cells had previously been exposed to 750  $\mu$ M t-BHP/Fe<sup>2+</sup> oxidant system, were performed. Since AA uptake is a rapid process in U937 cells (Fig. 7.1), uptake assays post-oxidation were conducted only for 4 h because an appreciable amount of <sup>3</sup>H-AA was quantified in cellular phospholipids at that time (Fig. 7.3). Findings indicate that as the incorporation of <sup>3</sup>H-AA into cellular phospholipids increases the rate of uptake decreases over time.

Even under normal incubation conditions (in resting cells) without any form of activation, the capacity of U937 cells to incorporate AA into phospholipids by the reacylation pathway is high (Fig. 7.1). For AA incorporation to occur, there must be LPL acceptors available, most likely by PLA<sub>2</sub> hydrolytic activity. Thus, it can be said that U937 cells possess a basal PLA<sub>2</sub> activity, enough to account for AA incorporation abilities. As different isoforms of PLA<sub>2</sub> including cPLA<sub>2</sub> (Kramer et al., 1991) and iPLA<sub>2</sub> (Hsu et al., 2000) have been identified in U937 cells, under resting conditions, the exact PLA<sub>2</sub> enzyme providing lysophospholipid acceptors for arachidonic acid reacylation into phospholipids is yet unknown. However kinetic studies of AA uptake

by U937 cells demonstrated that  $^3\text{H}$ -AA incorporation was nearly completed after 24 h of incubation with about 10% of radioactivity remaining in the culture medium.

This study also demonstrated that U937 cells manifest a higher capacity to import exogenous  $^3\text{H}$ -AA into cellular phospholipids when subjected to oxidative stress (Fig. 7.2). The increase in AA incorporation into phospholipids increases dramatically for 1 h followed by a rapid decrease in uptake indicating that there is a modification in the substrate required and/or enzyme responsible for the incorporation of AA, under prolonged conditions of oxidation. When the cells were incubated with  $^3\text{H}$ -AA in fresh medium after induced-oxidation, cellular uptake continued over 24 h but at a higher rate in oxidant activated – U937 cells (Fig. 7.3).

#### **7.4.1 With fatty acids**

Unlabelled EPA treatment of U937 cells appeared to exert suppressive influences on the incorporation of labelled AA into cellular phospholipids (Figs. 7.4 and 7.5). Interestingly, DHA treatment gave the opposite effects. It is likely that the differences observed in the response of these PUFAs (EPA and DHA) to  $^3\text{H}$ -AA uptake reflects the competitive nature between the  $\text{C}_{20}$  PUFAs (AA and EPA) for the sn-2 position of glycerophospholipids compared to DHA as suggested by Balsinde (2002). Although it was previously reported that EPA is preferentially taken up into PE over PC and AA into PC in U937 cells (Balsinde, 2002) it was also reported that PUFAs particularly AA, migrate between these phospholipid classes over time (Balsinde et al., 1994; Balsinde, 2002).

Without induced-oxidative stress, co-treatment of U937 cells for 24 h with the radiolabel and AA or EPA inhibit  $^3\text{H}$ -AA uptake while DHA does not, when compared

to controls (Fig. 7.4). Suppression of  $^3\text{H}$ -AA incorporation by unlabelled AA or EPA but not DHA is proposed to reflect reduction in lysophosphatidyl choline (LPC) acceptor molecules, the abundance of which is thought to limit the rate of AA incorporation and to be governed by PLA<sub>2</sub> activity. Independent of the conditions employed, treatment of U937 cells with EPA appeared to have more inhibitory effects (sometimes insignificant as in Fig. 7.6) than DHA on  $^3\text{H}$ -AA uptake into cellular phospholipids. This decrease in  $^3\text{H}$ -AA uptake was definitely not due to loss of membrane integrity because over the 18 d period of continuous FA treatment, cell viability was > 90% in all treatments as monitored by the Trypan Blue dye-exclusion method. Since all FFAs were washed off before oxidation, it appears likely that EPA but not DHA in the esterified form, strongly competes with AA for the sn-2 position of phospholipids. However, this does not explain why  $^3\text{H}$ -AA uptake is more pronounced after extended periods of EPA-treatments. Another possible explanation is that EPA acts by inhibiting enzymes such as the acyltransferase, responsible for the reacylation of AA into phospholipids whilst DHA does not.

In a recent study of the phospholipid fraction in rat uterine stomal cells (U<sub>111</sub> cells), Luquain et al (2000) found that DHA but not AA or EPA, was selectively esterified extensively into alkenylacyl-glycerophosphoethanolamine (PE-plasmalogens) as well as a minor phospholipid designated as bis-(monoacylglycerol phosphate). In that study, the percentage of labelled DHA quantified in PC and PS/PI reduced rapidly with a concomitant increase in BMP and PE. It is therefore possible that the high uptake of  $^3\text{H}$ -AA observed with DHA-treated cells, similar to that obtained with SA or OA, was due to the rapid incorporation of the added free  $^3\text{H}$ -AA into LPC molecules poor in PUFAs, generated by a PLA<sub>2</sub> enzyme in response to the induced oxidative stress.

Upon continuous FA treatments, the inhibitory effects of AA and EPA were pronounced whereas DHA – treated cells appeared to incorporate the most  $^3\text{H}$ -AA in comparison to the other two PUFAs. As shown in Fig 7.7, the incorporation of  $^3\text{H}$ -AA after induced-oxidation was not affected by prolonged treatment of U937 cells with SA, OA or even DHA but was severely suppressed by the presence of unlabelled AA and EPA. These results are in support of previously reported studies in U937 cells (Balsinde, 2002) and mouse peritoneal macrophages (Fernandez et al., 1992). A possible reason for differences in  $^3\text{H}$ -AA uptake is that PLA<sub>2</sub> activity may be subject to different mechanisms of regulation depending on the PUFA involved.

The differences observed with the different fatty acids employed, namely: stearic, oleic and most especially the polyunsaturated fatty acids: arachidonic, eicosapentaenoic and docosahexaenoic acids, will be discussed in more detail at the end of this study.

#### **7.4.2 Antioxidants**

To determine the role of dietary antioxidants on oxidant-induced  $^3\text{H}$ -AA incorporation into cellular phospholipids, the effects of pre-incubating U937 cells with ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene or astaxanthin coupled with AA, EPA or DHA were examined. Endogenous presence of ascorbic acid in U937 cells subjected to oxidative stress significantly reduced the uptake of  $^3\text{H}$ -AA into cellular phospholipids. Since ascorbic acid is capable of promoting iron-dependent oxidative damage (Winterbourn, 1981; Minetti et al., 1992), the decrease in AA incorporation may be due to the presence of a large proportion of inactive U937 cells with lost membrane integrity. Although the pro-oxidative effect of ascorbic acid was not obvious in chapter four (FOX assay) after induced-oxidation for 30 min, it should be noted that AA uptake assays were conducted



after twice that length of time (1 h), suggesting a time-lag phase for the peroxidative attribute of the ascorbate radical.

On the other hand,  $\beta$ -carotene and astaxanthin appeared to reduce the uptake of  $^3\text{H-AA}$  with AA or EPA-pre-treated cells but not with DHA whereas,  $\alpha$ -tocopherol showed no effect on  $^3\text{H-AA}$  uptake in oxidised U937 cells. A possible explanation for the failure of  $\alpha$ -tocopherol to influence fatty acid incorporation into phospholipids may be due to factors such as the location of the vitamin in the membrane, the rate and site of the peroxy radical production and the form of interaction existing between the lysophospholipase enzyme, acyltransferases and the cell membrane post-oxidation. Likewise, the location of carotenoids in U937 cell membranes could affect their ability to function as antioxidants.

Since the phenolic moiety of  $\alpha$ -tocopherol is likely oriented towards aqueous solutions, it can react with a water-soluble initiating radical to form the stable phenoxyl radical (Matsuo et al., 1989; Liebler et al., 1991). Since the phenoxyl radical must also terminate itself, it could react with an unoxidized lipid molecule instead, thus propagating peroxidative chain reactions in the process (Dugas et al., 1998). In an attempt to enrich U937 cells *in vitro*,  $\beta$ -carotene and astaxanthin, may have been incorporated only near the surface and not necessarily in positions where they might be normally located *in vivo* (Borel et al., 1996). To this end, it is important to extend these studies to *in vivo* enrichments of these dietary antioxidants to confirm the results presented here.

### 7.4.3 Conclusion

In summary, from this study, it is obvious that oxidation plays an important role in the modulation of phospholipid fatty acid turnover by providing LPL acceptors required for the reacylation reaction. In addition, results herein demonstrated that  $^3\text{H}$ -AA uptake is significantly dictated by the identity of the fatty acid composition already present in U937 cells. Whilst SA or OA does not influence  $^3\text{H}$ -AA incorporation, the PUFAs are involved in the determination of synthetic process of the formation of cellular phospholipids.

Unlike the saturated fatty acids, AA and other PUFAs such as EPA do not generally enter cellular phospholipids by direct acylation via the *de novo* pathway, instead, at a later stage through direct acylation of previously-existing lysophospholipid (LPL) acceptors. Since LPLs are produced by the lipolytic action of  $\text{PLA}_2$  on phospholipids, this enzyme must play a significant role in the uptake of AA into phospholipids. However, much work is required to clarify the mechanisms responsible for LPL maintenance and the role of oxidation in phospholipid metabolism.

## CHAPTER 8: PRELIMINARY CYTOSOLIC PHOSPHOLIPASE A<sub>2</sub> (cPLA<sub>2</sub>) ELISA

### 8.1 INTRODUCTION

Cells *in vivo* must continuously adapt to stress stimuli such as free radicals and highly reactive oxygen species. In schizophrenia, the biochemical details underlying the relationships between oxidative injury and PLA<sub>2</sub> activity subsequently leading to neurodegeneration, are yet to be clarified. PLA<sub>2</sub>s are a growing family of enzymes including a heterogenous collection of proteins with diverse roles in cell functions (Six and Dennis, 2000). Among this group is cPLA<sub>2</sub>, a cytosolic enzyme catalysing the hydrolysis of phospholipids at the sn-2 position generating free fatty acids, preferentially arachidonic acid (AA) and lysophospholipids. The release of AA is particularly relevant because it is the common precursor of the biologically active eicosanoids (Smith, 1992). AA and its metabolites can also act as first and second messengers in the modulation of a number of cellular processes (Axelrod et al., 1988). Therefore, the regulation of an AA-specific PLA<sub>2</sub> serves as a key control point for the synthesis of potent mediators of inflammatory responses and signal transduction pathways.

The 85 kDa cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) that migrates with an apparent molecular mass of ~100 kDa (cPLA<sub>2</sub>), is expressed in a variety of cell types including the human monocytic cell line, U937 (Clark et al., 1990; Kramer et al., 1991; Sharp et al., 1991). The molecular details responsible for the signaling pathways of cPLA<sub>2</sub> are thought to involve its phosphorylation (Channon and Leslie, 1990; Clark et al., 1991). One well-characterized mechanism of agonist-stimulated cPLA<sub>2</sub>-enzyme activation involves increased intracellular concentrations of Ca<sup>2+</sup> (Channon and Leslie, 1990; Gijon et al., 1999) leading to its phosphorylation *in vitro* at a serine (Ser-505) amino acid residue (de Carvalho et al., 1996; Gijon et al., 1999). The observation that a large fraction of cPLA<sub>2</sub>s can be found phosphorylated even under basal or non-stimulated conditions, clouds the role of increased

phosphorylation of cPLA<sub>2</sub> towards cellular agonist activation (Lin et al., 1993; Gijon et al., 1999; Gijon and Leslie, 1999). In addition, it has been shown that cPLA<sub>2</sub> has a high affinity for phosphatidylinositol 4, 5-bisphosphate (PIP<sub>2</sub>) in cell membranes (Mosier et al., 1998). Furthermore, studies have shown that cPLA<sub>2</sub> can be activated to generate arachidonic acid (AA), without any increase in intracellular Ca<sup>2+</sup> concentration (Balsinde et al., 2000).

Another pathway implicated in cPLA<sub>2</sub> activation involves the coupling of cPLA<sub>2</sub> to receptors via G-proteins (Burch, 1989) and cPLA<sub>2</sub> phosphorylation activated *in vivo* by a stimulated protein kinase C (PKC) in response to cell activation (Parker et al., 1987; Lin et al., 1993; de Carvalho et al., 1996; Gijon et al., 1999). There is growing evidence of the involvement of cPLA<sub>2</sub> in receptor-mediated generation of eicosanoids and platelet activating factors in several pathological processes (Larsen and Henson, 1983) including the modulation of ion channels (Kim and Clapham, 1989) and neurotransmitter release (Piomelli and Greengard, 1990).

The activation of cPLA<sub>2</sub> by increased Ca<sup>2+</sup> concentrations causes its translocation from the cytosol to the plasma membranes where the phospholipid substrate is localized (Channon and Leslie, 1990; Clark et al., 1991). Since oxidative injury is capable of altering membrane permeability and fluidity by modulations of cell membrane associated proteins such as the ion channels, its implication in neurodegeneration (Farooqui and Horrocks, 1991) is no surprise. It has been shown that PLA<sub>2</sub> activity is increased in schizophrenia (Gattaz et al., 1995) and this has been associated with increasing oxidative stress in neuronal cell membranes (Scheffer et al., 1999). It is likely that oxidative stress stimuli increase the phosphorylation of cPLA<sub>2</sub> above basal levels thereby, increasing its activity.

To further understand the role of oxidant-induced over-activity of cPLA<sub>2</sub>, this preliminary study investigates the concentration of the enzyme in U937 cells exposed to a free radical mediating oxidant system, using a novel double antibody “sandwich” enzyme-linked immunosorbent assay (ELISA).

An ELISA-based technique developed by the Biochemistry Department of the Victoria Infirmary NHS Trust (Glasgow) was used to measure cPLA<sub>2</sub> levels in oxidant-activated U937 cells. The assay employs antibodies, which recognize the cPLA<sub>2</sub> enzyme. The antibodies were used in this preliminary study to determine immunoreactivity of U937 cells treated with or without the oxidative stress stimuli after pre-incubations with the different fatty acids.

## **8.2 MATERIALS AND METHODS**

### **8.2.1 Materials**

Fatty-acid-free bovine serum albumin (BSA), stearic acid (SA), oleic acid (OA), arachidonic acid (AA), 0.4% Trypan Blue dye solution, Hanks balanced salt solution (HBSS), RPMI 1640, foetal bovine serum (FBS), 200 mM L-glutamine, 50 mg/ml gentamicin solution, HPLC-grade ethanol, tert butyl hydroperoxide, and ferrous sulfate heptahydrate (FeSO<sub>4</sub> · 7H<sub>2</sub>O) were obtained from Sigma-Aldrich, UK. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were supplied by Nu-chek Prep. Inc., Denmark.

### 8.2.2 Glassware and plastic preparations

All glassware used was washed in de-con and nano-pure water, autoclaved at 121°C for 30 min and dried in an oven before use. All other apparatus used were sterile with all U937 cell treatments performed in a class II laminar flow hood.

### 8.2.3 Cell culture

U937 cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine and 0.05 mg/ml gentamicin at 37°C in a humidified incubator with 5% CO<sub>2</sub>/95% air. For all experiments, cells were maintained at  $1.0 \times 10^6$  cells/ml at viability > 90% by Trypan Blue dye exclusion method of viability measurements.

### 8.2.4 Cell treatments

U937 cells adjusted to contain  $1.0 \times 10^6$  cells/ml were treated with the FAs: SA, OA, AA, EPA, DHA or 0.4% v/v of ethanol (controls) complexed with fatty-acid-free BSA in a 2.5:1 molar ratio. Cells were incubated in 1 ml suspensions in 24-well plates at 37°C and 5% CO<sub>2</sub> in a humid environment. Final concentration of fatty acids in 1.0 ml cell suspensions per well was 10 µM. After 24 h of incubation with FAs, cells were washed twice with HBSS/0.2% BSA by centrifugation at 250 g for 5 min at 4°C and re-suspended in 0.1 ml of HBSS in new 24-well plates. Stock solutions of 2 mM t-BHP and 5 mM FeSO<sub>4</sub> were freshly prepared in HBSS. Cells (0.1 ml in HBSS) were then treated with or without 0.9 ml of the oxidant mixture in HBSS. The final oxidant concentration in each well of a 24-well plate was 750 µM t-BHP/Fe<sup>2+</sup>. Cells treated without the oxidant mixture were incubated in HBSS only. Incubation proceeded for 1 h under the conditions stated above. Thereafter, reactions were stopped by centrifugation at 4000 g for 3 min at 4°C. Cells were washed

twice in HBSS and re-suspended in 0.1 ml of HBSS. Cell pellets (0.1 ml) in HBSS were frozen immediately at  $-200^{\circ}\text{C}$  in liquid nitrogen prior to the ELISA.

### **8.2.5 cPLA<sub>2</sub> ELISA technique**

Oxidant-treated cell samples were transported to the Biochemistry Department, Victoria Infirmary NHS Trust (Glasgow) in liquid nitrogen. On arrival, samples were transferred into a  $-70^{\circ}\text{C}$  freezer for storage. Cell samples were analyzed using an as yet unpublished ELISA technique developed in their laboratory (MacDonald et al., 2000). For this reason, the method will not be disclosed in this thesis.

### **8.2.6 Protein assay**

The protein content of cell homogenates and supernates were determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a reference standard.

## **8.3 RESULTS**

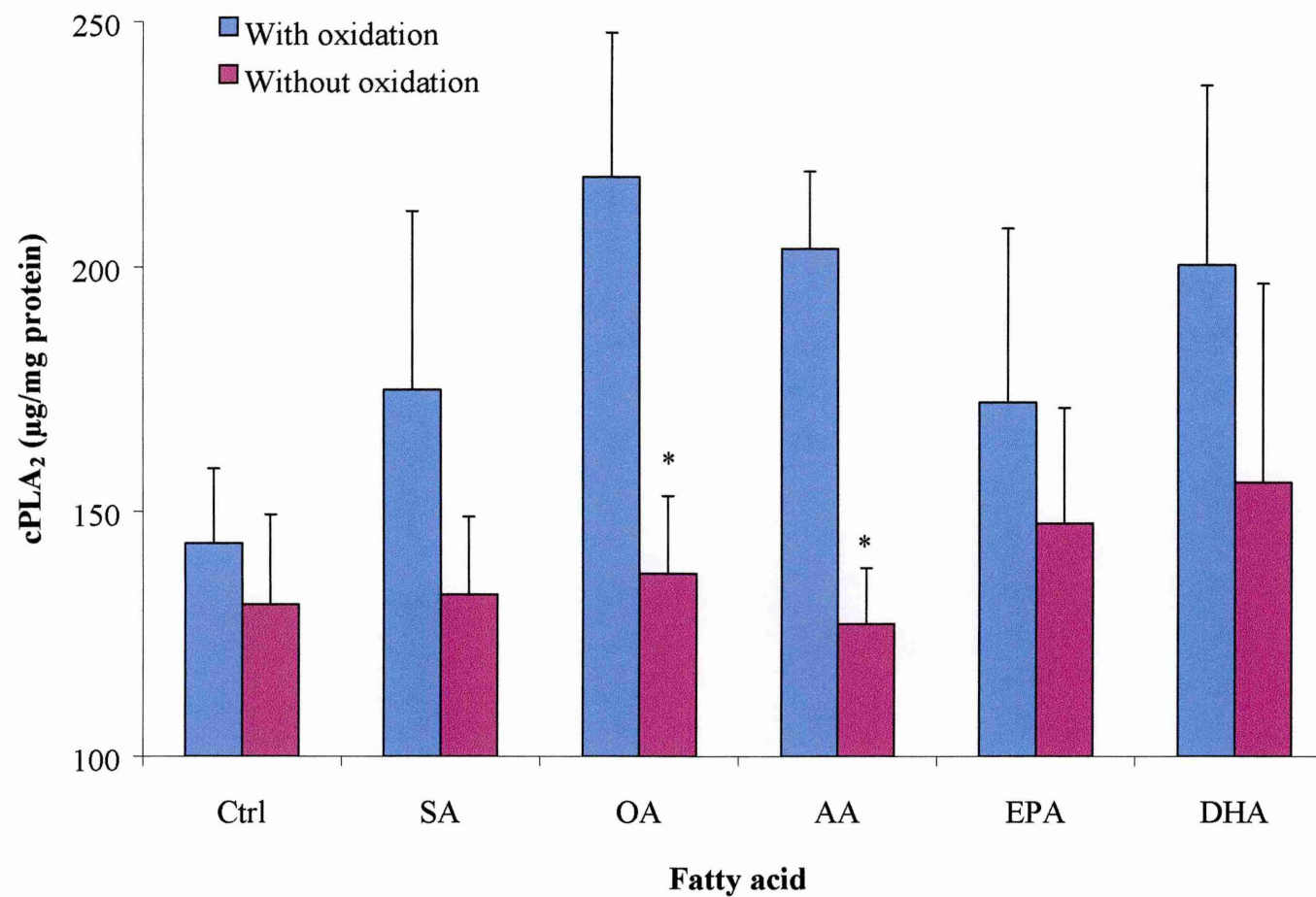
To determine the effect of the different fatty acids on cPLA<sub>2</sub> activity, U937 cells were incubated in Hanks balanced salt solution (HBSS) only for 1 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in a humid environment. The ELISA technique for the measurement of the concentration of cPLA<sub>2</sub> was used as a measure of the concentration of the enzyme. U937 cells pre-treated with 10  $\mu\text{M}$  levels of SA, OA, AA, EPA, DHA or 0.4% v/v ethanol (control) for 24 h were washed twice to rid cells of unincorporated FAs prior to the exposure of cells to HBSS (without oxidation) or 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  (with oxidation). Post-oxidation, cells were washed again twice in HBSS to ensure the removal of all serum before the conduction of the ELISA. No significant difference in cPLA<sub>2</sub> concentration with fatty acid treatments in cells treated with or without the oxidant mixture was observed (one-way ANOVA  $F = 2.35$ ,

$P > 0.05$ ,  $DF = 5$ ) (Fig. 8). To examine the overall effect of oxidative stress on cPLA<sub>2</sub> activity, U937 cell response was compared between cells treated with and without the oxidant mixture. Statistical analyses show a significant increase in cPLA<sub>2</sub> activity with oxidative stress (one-way ANOVA  $F = 31.92$ ,  $P < 0.001$ ,  $DF = 1$ ) (Fig. 8).

Comparisons of each set of U937 cells pre-incubated with fatty acids (treated with or without  $750 \mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$ ), showed no significant increases in cPLA<sub>2</sub> levels with induced-oxidation: controls ( $T = 1.03$ ,  $P > 0.05$ ,  $DF = 6$ ); SA- ( $T = 2.06$ ,  $P > 0.05$ ,  $DF = 6$ ); EPA- ( $T = 1.04$ ,  $P > 0.05$ ,  $DF = 5$ ) and DHA- ( $T = 1.59$ ,  $P > 0.05$ ,  $DF = 6$ ) pre-treated U937 cells. However, there were significant increases in cPLA<sub>2</sub> levels with OA ( $W = 26.0$ ,  $P < 0.05$ ,  $DF = 6$ ) and AA ( $T = 7.70$ ,  $P < 0.001$ ,  $DF = 6$ ) pre-treatments. In oxidant-treated U937 cells, cPLA<sub>2</sub> levels increased by average values of 9% (controls); 24% (SA); 37% (OA); 38% (AA); 14% (EPA) 22% (DHA) when compared to counterpart FA-treated cells without the oxidant mixture.

Since cPLA<sub>2</sub> has a preference for AA - containing phospholipids, the marked oxidant-activated increase in cPLA<sub>2</sub> levels observed with AA enrichment of U937 cells is not surprising. It is however interesting that oxidation of the other PUFA (EPA or DHA) – pre-treated U937 cells demonstrated no significant elevations in cPLA<sub>2</sub> concentration. A possible explanation is that the large errors in this experiment may be disguising the effects of oxidation on cPLA<sub>2</sub> concentration with EPA and DHA treatments.





**Fig. 8** cPLA<sub>2</sub> concentration in U937 cells pre-treated with 10 µM SA, OA AA, EPA, DHA or ethanol (for controls) prior to exposure to 750 µM t-BHP/Fe<sup>2+</sup> for 1 h. Data points represent means ± 95% CIs (n = 4). \* Denotes significant differences between FA-enriched cells treated with and without the oxidant.

## 8.4 DISCUSSION AND CONCLUSION

The aim of this study was to determine the effect of oxidation on cPLA<sub>2</sub> concentration preliminarily, in fatty acid – enriched U937 cells. The data obtained are consistent with elevated cPLA<sub>2</sub> levels in response to increased oxidative stress.

In order to measure the effect of oxidative-stress on the concentration of cPLA<sub>2</sub>, the enzyme was immunoassayed from oxidized or unoxidized U937 cells pre-treated with or without the different fatty acids: SA, OA, AA, EPA or DHA. Afterwards, cPLA<sub>2</sub> was quantified. Under basal conditions (resting U937 cells), the concentration of cPLA<sub>2</sub> was high relative to FA-unsupplemented controls. In fact, there were no significant differences between controls and FA-enriched cells under both conditions investigated (with or without oxidation). With induced-oxidation however, cPLA<sub>2</sub> concentrations were increased. These elevations in cPLA<sub>2</sub> concentrations were significant only with OA- and AA- pre-treated U937 cells but not with controls, SA-, EPA- or DHA- treated cells. EPA and DHA are PUFAs, highly susceptible to peroxidative damage. It is likely that the presence of these PUFAs, promote cPLA<sub>2</sub> activation in U937 cells in a manner that does not require the phosphorylation of the enzyme nor alterations in its concentration as previously suggested by Hsu et al., (2000). Or perhaps pre-treatment with EPA or DHA stabilizes cPLA<sub>2</sub> in U937 cell membranes under such conditions of oxidative stress.

In support of this study, Hatanaka (1996) previously showed a link between the formation of free radicals and activation of PLA<sub>2</sub> in primary neuronal cell cultures. Besides, it has been suggested that oxidized phospholipids are better substrates for cPLA<sub>2</sub> activity than the native phospholipid itself, demonstrating a repairing role for cPLA<sub>2</sub> in the removal of damaged lipids from cell membranes (McLean et al., 1993).

#### **8.4.1 Conclusion**

In conclusion, this preliminary study shows an association between oxidative stress, cellular phospholipid fatty acid composition and increased cPLA<sub>2</sub> activity although the exact mechanism of cPLA<sub>2</sub> activation upon oxidation is yet to be fully understood. However, in view of the potential importance of these findings in schizophrenia research, this study needs to be repeated in other cell models. Clearly, further work is required to validate the hypothesis of increased cPLA<sub>2</sub> activity with elevated oxidative stress levels.

## **CHAPTER 9: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PROSPECTS**

### **9.1 INTRODUCTION**

In the "Discussion and Conclusion" sub-sections at the end of previous experimental chapters, the findings most relevant to the objectives set out to achieve were highlighted. The main goal of this section is to link the observations made from the different studies together in order to form an overall picture of the various effects of the cell treatments employed and the relationships existing between these treatments and the responses triggered using the human monocytic, leukemia U937 cell line.

Several methods were utilized in the investigation of the effects of oxidants (a mixture of tert-butyl hydroperoxide and  $\text{Fe}^{2+}$ ) on cell membrane integrity, modified cellular phospholipids, accumulation of certain by-products of lipid peroxidation (lipid hydroperoxides and volatile hydrocarbons) and arachidonic acid release and uptake in the hydrogen peroxide-producing U937 cell line. These methods provided more information on the cytotoxic levels of certain fatty acids, especially the PUFAs (AA, EPA and DHA), and dietary antioxidants (ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin) in these cells. It also demonstrated the mechanisms possibly governing the response of U937 cells pre-treated with these agents upon induced-oxidation.

### **9.2 CELL VIABILITY MEASUREMENTS**

This study showed that U937 cells are fast growing cells when maintained at  $0.5\text{--}1.0 \times 10^6$  cells/ml. In addition to demonstrating a good linear relationship between the Trypan blue dye-exclusion and lactate dehydrogenase methods of viability measurements, this study has shown that U937 cells are more sensitive to the PUFAs than the saturated (SA) and monounsaturated (OA) fatty acids. Of the PUFAs used, EPA was the most toxic to U937 cells when supplemented with CM at equal concentrations before cellular incubations at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in a humid environment.

Neither SA nor OA had any effect on LDH leakage whereas the addition of equivalent concentrations of the PUFAs did. Further research is required for a better biochemical understanding of mechanisms by which the PUFAs especially EPA, influence the loss of cell membrane integrity, without oxidative stress.

Initial studies demonstrated that the enrichment of these cells with 100 – 400  $\mu\text{M}$  levels of ascorbic acid, 10 – 40  $\mu\text{M}$  of  $\alpha$ -tocopherol, 3 – 12  $\mu\text{M}$  concentrations of  $\beta$ -carotene or astaxanthin did not alter cell membrane integrity significantly, establishing the tolerable concentrations of these agents in U937 cells prior to stress experiments with oxidants.

### 9.3 FATTY ACID INCORPORATION

Preliminary experiments by gas chromatographic analysis of fatty acids after pre-incubation of cells in FA-enriched CM demonstrated that U937 cells rapidly incorporate PUFAs into phospholipids. Data obtained suggest that these cells are rich in saturated (14:0, 16:0 and 18:0) and monounsaturated (16:1n-7, 18:1n-7 and 18:1n-9) fatty acids but very poor in the PUFAs (20:4n-6, 20:5n-3 and 22:6n-3) (North et al., 1994; Obermeier et al., 1995; Yano et al., 2000). However, supplementation with the PUFAs significantly increased their levels in cellular phospholipids after only 12 h of incubation. For fatty acid incorporation into phospholipids to occur, there must be pre-existing LPL acceptors. Since LPLs are generated by the hydrolytic action of  $\text{PLA}_2$  on phospholipids, this class of enzymes necessarily play a critical role in available-PUFA incorporation into cellular phospholipids in these cells, thereby demonstrating the existence of basal acyltransferase, lysophospholipase and phospholipase  $\text{A}_2$  activity under normal conditions of incubation. Previous studies have shown that U937 cells are rich in different isoforms of  $\text{PLA}_2$  including  $\text{cPLA}_2$  (Kramer et al., 1991) and  $\text{iPLA}_2$

(Balboa and Balsinde, 2002), the exact mechanism therefore responsible for FA incorporation under resting conditions requires further investigation.

## **9.4 EFFECTS OF THE FATTY ACIDS AND DIETARY ANTIOXIDANTS ON LIPID PEROXIDATION AND PHOSPHOLIPASE A<sub>2</sub> ACTIVITY**

The initial objectives and hypothesis outlined in chapter one will now be discussed in relation to the results obtained in this research in order to answer the following questions:

- Is oxidative stress measurable in U937 cells?
- Does oxidative stress increase the uptake of fatty acids into phospholipids (PLs) or the breakdown of PLs?
- How do the PUFAs (EPA and DHA) affect the release and uptake of AA under oxidative stress conditions?
- Is the expression of PLA<sub>2</sub> influenced by oxidative stress?
- What are the effects of the antioxidants (ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin) on oxidative stress and PLA<sub>2</sub> activity in PUFA-rich U937 cells?
- Is lipid peroxidation coupled with PLA<sub>2</sub> activation in U937 cells?

### **9.4.1 Is oxidative stress measurable in U937 cells?**

Lipid peroxidation is a critical cellular process that occurs in aerobic organisms in response to oxidative stress. This process places a burden on the whole cell or organ and has been studied both in *in vivo* and *in vitro* animal model systems. The Ferrous oxidation/xlenol orange (FOX) assay was used to determine the susceptibility of U937 cells to lipid peroxidative damage after fatty acid modifications of their cellular phospholipids. This study showed that fatty acids oxidize at different rates, yielding lipid peroxides, in a manner dependent on their degree of unsaturation, when examined

under conditions of equal oxidative stress. For confirmatory reasons, U937 cells pre-treated with the same fatty acids were tested for volatile hydrocarbons (alkanes) generated post-oxidation by headspace analysis. In contrast to expectations, U937 cells generated ethane and butane in a decreasing fashion and pentane in an increasing manner, with increasing levels of FA unsaturation. Since hydrocarbons are metabolised by the decomposition of lipid peroxides (Halliwell, 1987; De Zwart et al., 1999), the mechanism responsible for the production of lower levels of ethane and butane from cells pre-treated with PUFAs especially EPA and DHA, in comparison to SA and OA, is not yet clear. Likewise, the system by which these cells generate pentane with the different PUFAs is not yet understood. A possible explanation may be that fatty acids oxidize at different rates, producing different by-products of lipid peroxidation, in a way independent of unsaturation levels, as previously suggested by Visioli et al. (1998). It may also be that there is a lag phase between ethane production from lipid peroxides in FA-treated cells upon induced oxidation. Whether the generation of volatile hydrocarbons from FA- pre-treated cells involves an enzyme activity is unknown at present.

Taken together, this study shows that lipid peroxides and volatile alkanes (ethane, butane and pentane) can be quantified in U937 cells as biomarkers of lipid peroxidation. However, whether the technique of GC-headspace analysis of volatile hydrocarbons can be proposed as a suitable method of analysing by-products of lipid peroxidative damage in the U937 cell line requires much further investigation. Also, it suggests that several biomarkers of lipid peroxidative damage should be measured when studying the effects of treatments of oxidative stress whether *in vitro* or *in vivo*.

#### **9.4.2 Does oxidative stress increase the uptake of fatty acids into phospholipids (PLs) or the breakdown of PLs?**

The radical-mediating system of 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  was used in this study to investigate the release and incorporation of radiolabelled AA. This concentration had a significant effect on U937 cell membrane integrity compared to controls. Experiments indicated that AA release in U937 cells exposed to the oxidants is both dose- and time-dependent. In resting U937 cells, the capacity to incorporate AA into phospholipids is high. Whilst prolonged oxidation ( $> 1$  h) of untreated cells decreased the uptake of radiolabelled AA, short exposure ( $\leq 1$  h) to 750  $\mu\text{M}$  t-BHP/ $\text{Fe}^{2+}$  significantly increased the rate of incorporation into cellular phospholipids.

Increasing evidence suggests that free radical-mediated damage contributes directly or indirectly to schizophrenia (De Zwart et al., 1999). The present study showed a significant relationship between the generation of lipid hydroperoxides, the reacylation of AA into cellular PLs and the hydrolysis of phospholipids, yielding second messengers: arachidonic acid and lysophospholipids (LPLs). LPLs are potentially toxic in nature due to their detergent-like properties and in high concentrations, both AA and LPLs can cause membrane disruption and ultimately cell lysis (Farooqui et al., 1997b).

#### **9.4.3 How do the PUFAs (EPA and DHA) affect the uptake and release of AA under oxidative stress conditions?**

The polyunsaturated fatty acids (PUFAs), especially EPA and DHA, are being increasingly investigated as possible treatments for a diverse array of human diseases. These include: atherosclerosis (Gil, 2002), cancer (Cowing and Saker, 2002), cardiovascular diseases (Nordoy et al., 2001), schizophrenia (Horrobin et al., 2002), depression (Puri et al., 2001) and many neurodegenerative disorders (Blondeau et al.,



2002). These reports emphasize the importance of the PUFAs (both n-3 and n-6) as basic biomolecules in biological systems. Of the n-3 PUFAs, supplementary treatment with EPA but not DHA significantly improves symptoms of schizophrenia (Emsley et al., 2002). A number of points from the present study indicate similarities and striking differences in the response of U937 cells to oxidative stress post treatments with EPA or DHA for varying periods of time:

- EPA use, above certain tolerable concentrations, has stronger cytotoxic effects on U937 cell membrane integrity compared to DHA when used in equal concentrations and cells incubated for 3 days.
- At equal concentrations, there were no significant differences between the susceptibility of EPA and DHA pre-treated cells (for 24 h) to peroxidative damage as quantified by both the FOX assay and headspace analysis. Accumulation in lipid hydroperoxides increased with increasing concentrations of both EPA and DHA.
- Neither EPA nor DHA inhibited oxidation under the conditions studied. Increasing concentrations of EPA or DHA promoted the release of radiolabelled AA into the extracellular medium when exposed to the oxidants.
- In an attempt to gain further insight into the mechanisms influencing the response of U937 cells under oxidative stress conditions, the fatty acids (SA, OA, AA, EPA or DHA) were continuously supplemented with the culture medium (by complete replacement every 3 days) for 18 days before oxidation experiments for the determination of AA release and AA uptake were conducted. Interestingly, there was a marked difference in AA release and uptake between cells treated with EPA and DHA. EPA strikingly promoted AA release and inhibited its incorporation into phospholipids in comparison to DHA-treated cells under oxidative stress conditions. This unexpected finding of

suppressed AA uptake with induced-oxidation indicates that prolonged EPA pre-treatment totally reversed the stimulatory effects of oxidative stress observed in FA-untreated control cells. Whether such a response reflects an effect of EPA alone or an influence of the fatty acid triggered by the peroxidative process, is unknown at present and should be a subject for future investigations. This information will be provided by repeating the experiment with controls for the prolonged fatty acid treatment including FA-treated cells alone without oxidative stress. It is however important to note that assays were conducted after pre-treating cells for 24 h with FAs and  $^3\text{H}$ -AA without oxidation (for AA release) (Fig. 6.4) and also after the simultaneous addition of FAs and  $^3\text{H}$ -AA for 24 h without oxidation (for AA uptake) (Fig. 7.4). Based on these results, it is tempting to speculate that after continuous treatments of these cells with the fatty acids for 18 days, in the absence of the oxidants, AA release remains significantly higher and uptake markedly lower with EPA-treatments in comparison to DHA-treated cells. The 24 h assays without induced-oxidation suggest that these effects result from EPA treatment alone, not requiring the oxidant to become apparent. Further studies are therefore required to investigate this attractive possibility.

Arachidonic acid (AA) is predominantly generated through the action of  $\text{PLA}_2$  by the hydrolysis of membrane phospholipids. Due to the pivotal role of  $\text{PLA}_2$  in the liberation of AA, the roles of SA, OA, AA, EPA and DHA in its release upon oxidant stimulation were investigated. Neither SA nor OA demonstrated any significant effects on AA release. Of the PUFAs employed, EPA demonstrated dramatically different and significant effects when continuously supplemented with the culture medium over 18 days. It appears likely that eicosapentaenoic acid stimulates the release of free AA, at

the same time inhibiting its uptake into cellular phospholipids after the exposure of the cells to t-BHP/Fe<sup>2+</sup>. This conclusion argues against my initial hypothesis that EPA acts as an antioxidant, since t-BHP/Fe<sup>2+</sup> stimulates AA release in FA-untreated U937 cell controls. How this oxidant-facilitated catalysis of AA release by EPA presence occurs is presently unknown, but a possible explanation is that the presence of EPA in U937 cell phospholipids enhances the release of AA upon oxidation, thus ensuring its availability for eicosanoid production. Also, it could be that EPA acts by blocking the re-incorporation of AA into cellular phospholipids, upon induced oxidation. Of note however, is the lack of control experiments designed to show the effects of the continuous (18 days) treatments of U937 cells with these fatty acids in HBSS only (without oxidants). This information is required to determine whether the differences observed in AA uptake and release between EPA and DHA occur only under conditions of oxidant-stimulation or under resting conditions as well.

Eicosanoids are oxygenated derivatives of di-homo- $\gamma$ -linolenic acid (DGLA), AA, and EPA. The relative concentrations of these three fatty acids affect the type of eicosanoids produced because they compete for the cyclo-oxygenase (COX) and lipoxygenase (LOX) enzymes. Eicosanoids metabolised from AA are stronger modulators of cell functions than those produced by DGLA or EPA (Crawford, 1983). On the other hand, DHA is a substrate for neither the COX nor LOX enzymes, but this study suggests that it can reduce the synthesis of eicosanoids from AA by inhibiting the activity of a phospholipase A<sub>2</sub> enzyme. Schizophrenic patients show reduced flushing in response to oral or topical niacin (nicotinic acid) compared to healthy controls, which can now be examined by the skin patch test (Ward et al., 1998). This abnormality has been shown in medicated and unmedicated schizophrenic patients in a study conducted in India (Shah et al., 1999). The mechanism underlying the niacin skin flushing is

based on the synthesis of prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) from arachidonic acid via the cyclo-oxygenase pathway (Morrow et al., 1992). The niacin test provides further evidence of a dysfunction in lipid signalling mechanisms in schizophrenia. In turn, these results highlight the key role of EPA in modulating the availability of AA released for eicosanoid synthesis under oxidative stress. Whether this impairment occurs in brain cells as well as monocytes is not yet clear.

The polyunsaturated fatty acids (AA, EPA and DHA) are derived from the essential fatty acids: linoleic and linolenic acid. Due to their crucial roles in signal transduction processes in the central nervous system (CNS), the importance of these fatty acids in human health cannot be over-emphasized. This study gives an insight into the actions of these fatty acids under conditions of oxidative stress. Since U937 cells are naturally poor in AA, EPA and DHA (North et al., 1994; Obermeier et al., 1995; Yano et al., 2000), raising their levels in these cells by CM-supplementation demonstrates the effects one might expect to observe in schizophrenia because cells from schizophrenic patients have also been shown to have depleted levels of these fatty acids (Mahadik et al., 1996). Furthermore, oxidative injury is often associated with AA mobilization from cells such as endothelial cells, platelets and phagocytes. Thus, interactions between peroxidative damage and AA release are of particular importance in biological systems.

In an open study, the diets of 20 hospitalised schizophrenic patients with chronic symptoms were supplemented with EPA from fish oils on a daily basis for 6 weeks (Mellor et al., 1995). Over the trial period, improvements in schizophrenic symptoms were observed and these were associated with increased erythrocyte membrane concentrations of n-3 fatty acids. Of particular interest, was the case of a 31 year-old drug free patient with longstanding schizophrenia who showed an 80 – 85%

improvement after daily dietary supplementation with EPA for 6 months (Puri and Richardson, 1998). The present study might help to explain why EPA appears to play a role in the reduction of schizophrenic symptoms by enhancing AA release. It was suggested that fatty acids released by PLA<sub>2</sub> activity along with their oxygenated metabolites (eicosanoids), play an important role in the regulation of neurotransmitters such as dopamine (Ross, in press). Perhaps the release of AA, markedly enhanced by EPA treatment aids the generation of eicosanoids in schizophrenia, in turn improving psychosis (Ross et al., in press).

As shown in this study, the levels of fatty acids in tissues reflect dietary intake (Zheng et al., 2001). For instance, reports from studies of normal volunteers receiving EPA and DHA supplementation, showed significant increases in n-3 fatty acids and reductions in the n-6 fatty acid compositions in the lipid fractions of the plasma, platelets and red blood cell membranes over 2 – 3 months (Prisco et al., 1996; Vidgren et al., 1997). Studies over longer periods of time suggest that FA levels in cholesteryl esters reflect intake over 1 – 2 weeks; red blood cells, over 1 – 2 months; and adipose tissues over years of intake (Katan et al., 1997). Several studies have reported depleted levels of linoleic acid (18: 2n-6) (Vaddadi et al., 1986; Horrobin et al., 1989; 1991; Kaiya et al., 1991; Fischer et al., 1992; Yao et al., 1994; Glen et al., 1994; Peet et al., 1995; Vaddadi et al., 1996); arachidonic acid (20: 4n-6); (Vaddadi et al., 1986; 1989; Horrobin et al., 1989; 1991; Yao et al., 1994; Peet et al., 1995); eicosapentaenoic acid (20: 5n-3) (Fischer et al., 1992; Glen et al., 1994; Peet et al., 1995); docosapentaenoic acid (22: 5n-3) (Vaddadi et al., 1986; 1989); and docosahexaenoic acid (22: 6n-3) (Vaddadi et al., 1989; Bates et al., 1991; Horrobin et al., 1991; Fischer et al., 1992; Glen et al., 1994; Peet et al., 1995) in schizophrenic patients compared to healthy controls. Provided that daily intake is not excessive, n-3 fatty acids are generally regarded as being safe (< 3 g

per day) (Department of Health and Human Services, 1997). Whilst three of four double-blind trials of n-6 fatty acid supplementation of standard antipsychotics medication produced negative results (Fenton et al., 2000), trials with n-3 fatty acids yielded positive results (Mellor et al., 1995; Peet et al., 1996), further suggesting the importance of the PUFAs especially the n-3 FAs in improving schizophrenic symptoms.

Most PUFAs used by neuronal cells for glycerophospholipid synthesis are not synthesized in the CNS but are transported to the brain from the gastrointestinal tract (Horrocks and Yeo, 1999). They are either dietary or produced in the liver from linoleic and linolenic acids. The synthesis of glycerophospholipids takes place in the endoplasmic reticulum (ER). Newly formed phospholipids self-assemble into thermodynamically stable bi-layers, which form vesicles that detach from the ER and then travel by phospholipid transfer proteins (Alb et al., 1996) to other sites in order to donate their phospholipids to other membrane structures (Farooqui et al., 2000). This process utilizes ATP to overcome the concentration and electrical gradients between the inner and outer membranes. Glycerophospholipids in neuronal membranes are responsible for membrane fluidity, permeability, local curvature, molecular packing or hydration, ionic charge and reactivity to regulate the activities of membrane-bound enzymes and ion-channels (Crews, 1982; Freysz et al., 1982). Therefore, the vitality of fatty acids in life cannot be stressed enough. Furthermore, populations with a high dietary intake of n-3 fatty acids were reported to show lower frequencies of breast cancer (Kaizer et al., 1989) and several studies have reported reduced multiplication of malignant cultured cells with n-3 fatty acid supplementation (Chow et al., 1989; Rose and Connolly, 1991; Anel et al., 1992; Chajes et al., 1995).

Deficiency as well as excess of PUFAs can influence the extent of cell death (Rudolf et al., 2001). The present study indicates that the effects of the n-3 fatty acids, EPA and DHA, ranges from stimulation to inhibition when U937 cells were exposed to oxidants. Interestingly, EPA enhances AA release over DHA and also reduces its incorporation when used at equal concentrations under conditions of oxidative stress, thus suggesting the involvement of a PLA<sub>2</sub> enzyme.

Collectively, this study has shown that over periods of continuous treatments, EPA acts by enhancing oxidant-induced AA release post AA uptake for 24 h and inhibiting <sup>3</sup>H-AA uptake after induced-oxidative stress. It can thus be argued that its mechanism of action involves the regulation of AA levels found incorporated into phospholipids as well as maintaining its availability for prostaglandin synthesis when exposed to oxidants. Recently clinical trials with ethyl-EPA generated greater improvements in schizophrenic symptoms when it produced elevations in AA levels and vice-versa (Horrobin et al., 2002). However, in that study, the total lipid extracts from red blood cells of patients analysed for fatty acids were assumed to contain mainly phospholipids. Further definitive assays were not employed to separate phospholipids from fatty acids after lipid extractions. Although it may be that the extracts mostly contained AA-rich phospholipids, they may also have contained cell-associated freely available arachidonic acid in response to EPA treatments. If the former is true, a possible explanation for increased AA release and suppressed uptake observed in this study with EPA treatments may be that the concentration of EPA used herein (10  $\mu$ M) is at the high end of the dose-response curve since clinical studies have shown that high amounts of EPA produces lower levels of incorporated AA into cellular phospholipids (Horrobin et al., 2002). It is also possible that at lower concentrations (< 10  $\mu$ M) EPA promotes AA release and does not inhibit its uptake under oxidative stress conditions. Hence it is

necessary to conduct dose-dependent experiments of AA release and uptake with or without oxidative stress after continuous pre-treatment of cells with EPA. It is important to note that the quantity of AA released/incorporated, though significantly higher/lower in EPA pre-treated cells is very low when compared to that still remaining incorporated in U937 cell phospholipids.

#### **9.4.4 Is the expression of PLA<sub>2</sub> influenced by oxidative stress?**

The specific PLA<sub>2</sub> enzyme responsible for stimulating AA release from phospholipids of U937 cells after the exposure to oxidants is not yet known. Although preliminary experiments by the ELISA technique of quantifying cPLA<sub>2</sub> expression in oxidant-stimulated and unstimulated U937 cells pre-treated with the different fatty acids seemed to show increased activity with oxidation, this increase was not significant between all FA-treatments (with and without oxidants). It is therefore possible that the sn-1 position of phospholipids is also extensively hydrolysed in U937 cells exposed to the oxidants, meaning that a PLA<sub>1</sub> could also be involved in regulating oxidant-induced AA reacylation into phospholipids. Other possibilities are: that the PLA<sub>2</sub> involved in oxidant-stimulated AA release may be a form of cPLA<sub>2</sub> (group IV), the group VI, intracellular PLA<sub>2</sub> (iPLA<sub>2</sub>) or even a yet unidentified isoform of PLA<sub>2</sub>. Of note, AA release experiments were conducted after 4 h of induced oxidation whereas the quantification of cPLA<sub>2</sub> by ELISA was performed after 1 h. It is therefore possible that cPLA<sub>2</sub> levels significantly increases in all FA treatments after 4 h rather than 1 h of exposure of U937 cells to the oxidant system.



#### **9.4.5 What are the effects of the antioxidants (ascorbic acid, $\alpha$ -tocopherol, $\beta$ -carotene and astaxanthin) on oxidative stress and PLA<sub>2</sub> activity in PUFA-rich U937 cells?**

Of all individual and mixtures of antioxidants used,  $\alpha$ -tocopherol was the most potent throughout this study. Although the carotenoids ( $\beta$ -carotene and astaxanthin) did not function effectively during extended conditions of induced-oxidation (4 h), their potency was demonstrated in situation of short exposure (30 min) to the oxidants. This is probably because they were consumed rapidly or due to the conditions of high oxygen tensions employed (Pryor et al., 1988). Whilst  $\alpha$ -tocopherol had no effect on AA uptake post-oxidation,  $\beta$ -carotene and astaxanthin conferred slight effects. Ascorbic acid on the other hand strikingly promoted AA release and markedly inhibited its uptake when oxidation was stimulated with the iron-containing oxidant system (t-BHP/Fe<sup>2+</sup>).

Taken together, the present study demonstrates that antioxidant activity is affected by the length of time of exposure of U937 cells to the oxidant. It is therefore necessary to conduct experiments involving the continuous treatment of U937 cells with these antioxidants over longer periods before induced-oxidation. It is important to note that different processes are affected differently by the various antioxidants utilized in the present study. This suggests that "monotherapy" with antioxidants may not confer the best effects but the use of mixtures of antioxidants from the diet, may produce better results than a pill of one type.

#### **9.4.6 Is lipid peroxidation coupled with PLA<sub>2</sub> activation in U937 cells?**

This study has shown that arachidonic acid is released in response to oxidative stress in U937 cells as previously demonstrated in this and other cell systems (Chakraborti and

Chakraborti, 1995; Birbes et al., 2000; Balboa and Balsinde, 2002) but the mechanisms responsible for this effect are not entirely known. Since assays of AA release with t-BHP/Fe<sup>2+</sup> were conducted for 4 h after several washings of the cells with HBSS containing BSA, data suggest that PLA<sub>2</sub> was activated in U937 cells in response to oxidation.

Interestingly, when these cells were subjected to oxidation prior to uptake experiments, results revealed an increase in AA incorporation for 1 h after which there was a marked decline in uptake suggesting AA accumulation in the extracellular fluid over time. This study found that U937 cells treated with t-BHP/Fe<sup>2+</sup> accumulate lipid peroxides over time in comparison to membranes from untreated cells. Taken together, the data suggest that the hydrolytic cleavage of cellular phospholipids by PLA<sub>2</sub> is enhanced in peroxide-treated cells probably due to changes in the physico-chemical state of U937 cell-membrane phospholipids in response to the accumulation of lipid peroxides, which was suppressed by the use of antioxidants such as  $\alpha$ -tocopherol.

## 9.5 GENERAL DISCUSSION

Based on all these results, it is hypothesized that the short exposure of cells to oxidants, leads to the accumulation of lipid peroxides which in turn increase the uptake of arachidonic acid into cellular phospholipids. However under extended periods of oxidative stress ( $\geq 4$  h), a decrease in AA incorporation is triggered due to the oxidation of membrane associated proteins (such as enzymes and receptors) concomitantly increasing extracellular concentrations of AA and LPLs, continuous accumulation of which eventually leads to cell death and neurodegeneration.

In schizophrenia, it is clear that there is a genetic component involved (Torrey, 1992; McGuffin et al., 1995). It is also widely accepted that there is considerable contribution by environmental factors such as the diet (Obi and Nwanze, 1979; Peet et al., 1995). Whilst not much is known about the distinctive symptoms of schizophrenia instantly distinguishing it from other psychiatric disorders such as bipolar disorder, a great deal remains to be discovered about the cause of the disease itself. Greden and Tandon (1995) suggested that the "knowledge gaps" in the long-term treatment of schizophrenia and other neurodegenerative diseases might be the most important issue facing psychiatry today based on the costs and consequences of maintaining improvements in severe psychiatric illnesses.

It is well known that early treatment of schizophrenia is clearly beneficial and that dangers are associated with untreated psychosis. Therefore the diagnosis and treatment at the onset of the disease seem imperative. The prevention of relapse is also a critical factor in the maintenance of schizophrenia treatment. Neuroleptics are associated with a variety of side effects and efforts to minimize these effects are necessary. Although the atypical antipsychotics cause less side effects, if the efficacy of EPA supplement with vitamin E in the treatment of schizophrenia is established by large clinical studies in humans, it may represent a new class of treatment for schizophrenia with much less baggage.

The above discussion on phospholipid metabolism, synthesis and breakdown, involving the PUFAs and dietary antioxidants, does not describe the entire dynamics of phospholipid activities in brain tissues, but instead provides further insights into the biochemical and molecular complexity of membranes. It is hoped that this study on the uptake and release of fatty acids will initiate further studies not only on the mechanisms

involved in oxidant-induced PLA<sub>2</sub> activity but also on individual activities of these fatty acids, their involvements in cell signalling and, ultimately, their roles in neuronal cell membranes.

## 9.6 CONCLUSIONS

In keeping with the above notions, preliminary studies with EPA but not DHA-treatments over time have yielded greater positive effects on the positive symptoms in schizophrenia in comparison to DHA (Peet et al., 2001). This finding is consistent with the possibility that the potency of individual n-3 PUFAs in altering cellular processes under conditions of oxidative stress, such as cell membrane leakage, AA release and uptake varies with PUFA type, concentration and time of exposure, as shown herein. It is noteworthy that the influence of PUFAs on these processes is possibly affected by the model system investigated and even the nature of examinations carried out whether *in vivo* or *in vitro*. It is not entirely clear whether n-3 PUFAs have direct influences on these cellular processes or if their metabolites are directly responsible.

In summary, this research has shown a strong relationship linking peroxidative damage, AA release and uptake in the presence of the various fatty acids to the loss of membrane integrity and ultimately cell death. Although pre-treatment with ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene and astaxanthin inhibited lipid peroxidation, only  $\alpha$ -tocopherol consistently conferred protective properties against oxidant-induced AA release from cellular phospholipids. Interestingly, none of the antioxidants at the concentrations used markedly influenced AA uptake post-oxidation. It is now important to apply this information in humans to determine whether the same effects are obtained.

## 9.7 FUTURE PROSPECTS

The clarification of the effects of continuous treatments of U937 cells with EPA and DHA alone without induced-oxidation on AA release and uptake will be needed to fully understand the mechanisms involved in the therapeutic effects of the n-3 polyunsaturated fatty acids.

Clearly, much work is yet to be done to clarify the mechanisms responsible for oxidant-induced AA release/uptake and the identification of the possible pathways and specific PLA<sub>2</sub> enzyme(s) involved. This will assist in the provision of the fundamental knowledge required for future research aimed at understanding the roles of PUFAs, especially EPA, in the treatment of schizophrenia and possibly other neurodegenerative diseases.

Taking all these findings together, it is attractive to speculate that cPLA<sub>2</sub> is responsible for the liberation of AA in oxidant-treated U937 cells. However, more work is needed to investigate this possibility. Due to the importance of acylation reactions in maintaining AA levels at physiological concentrations required for eicosanoid synthesis, it is necessary to confirm that oxidant-activated AA released with EPA treatments, actually yields eicosanoid production.

Recent data have highlighted the fact that proteins are oxidised first before lipids in U937 cells (Gieseg et al., 2000), which may mean that the PLA<sub>2</sub> enzyme responsible for catalysing AA release and uptake upon oxidation could be the oxidized form. Such a possibility as well as the involvement of EPA in enhancing oxidant-induced AA release requires further investigation. Further biochemical studies are also required to explore

the potential signalling mechanisms by which the PUFAs especially EPA, can act on gene expressions *in vivo*.

This study demonstrates that  $\alpha$ -tocopherol is more potent than  $\beta$ -carotene, astaxanthin and ascorbic acid against lipid peroxidative damage induced with t-BHP/ $\text{Fe}^{2+}$ . However, it is important to extend these studies (with individual and mixtures of antioxidants) to human subjects to confirm the results presented herein. The present study has shown that the polyunsaturated fatty acids exert pronounced effects in U937 cells in culture demonstrating their biochemical potentials as pharmacological agents.

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## APPENDIX: STATISTICAL ANALYSIS

Data are presented as the means  $\pm$  95% confidence intervals (CIs). Comparisons among normal groups of data (Anderson-Darling's test) with homogeneity of variance (Bartlett's test) were made by one-way analysis of variance (ANOVA) using MINITAB statistical software, version 13.32. When a significant effect was observed, *post hoc* comparisons of means were made using Tukey's pairwise comparisons. Differences were considered at  $P < 0.05$ .

In chapters 6 and 7 (radioactivity counts in dpm expressed as percentage values), when the evidence indicated normal groups of data but with heterogeneity of variance, thereby failing the tests for performing one-way ANOVA, square root transformations of percentage values were used. After these transformations, homogenous variances were obtained.

In situations where groups of data were not normally distributed (Anderson-Darling's test), but with equal variances (Levene's Test), comparisons were made by Kruskal-Wallis test using MINITAB release 13.32. This was followed by the Dunnett's test for multiple comparisons. P values less than 0.05 were considered significant.